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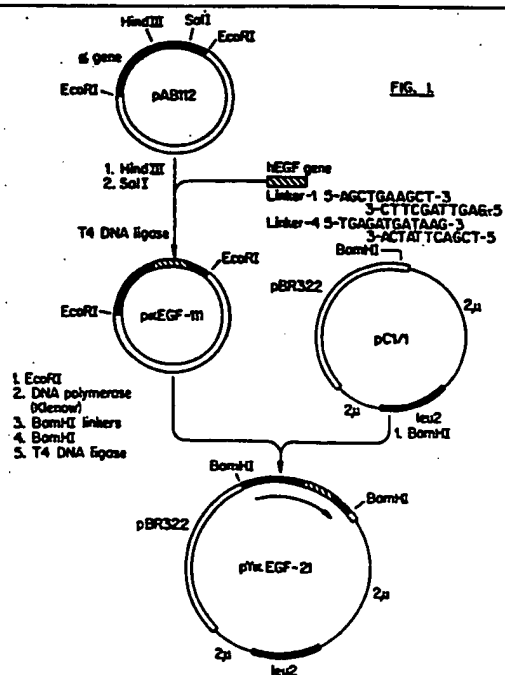
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(54) **Secretory expression in eukaryotes.**

(57) Methods and compositions are provided for producing polypeptide sequences in high yield by employing DNA constructs, wherein the DNA sequence encoding for the polypeptide of interest is preceded by a leader sequence and processing sequence for secreting and processing said polypeptide. In this manner, the mature polypeptide of interest may be isolated from the nutrient medium substantially free of major amounts of other proteins and cellular debris.

The yeast strain *S. cerevisiae* AB103 (pYEGF8) was deposited on January 5, 1983, at the A.T.C.C. and given accession No. 20658.

The plasmid pYαEGF23 (pAB114-pC1/1) was deposited at the A.T.C.C. on August 12, 1983, and given Accession No. 40079.



SECRETORY EXPRESSION IN EUKARYOTES

BACKGROUND OF THE INVENTIONField of the Invention

5 Hybrid DNA technology has revolutionized the
ability to produce polypeptides of an infinite variety
of compositions. Since living forms are composed of
proteins and employ proteins for regulation, the
ability to duplicate these proteins at will offers
10 unique opportunities for investigating the manner in
which these proteins function and the use of such
proteins, fragments of such proteins, or analogs in
therapy and diagnosis.

 There have been numerous advances in improv-
15 ing the rate and amount of protein produced by a cell.
Most of these advances have been associated with higher
copy numbers, more efficient promoters, and means for
reducing the amount of degradation of the desired
product. It is evident that it would be extremely
20 desirable to be able to secrete polypeptides of interest,
where such polypeptides are the product of interest.

 Furthermore, in many situations, the polypep-
tide of interest does not have an initial methionine
amino acid. This is usually a result of there being a
25 processing signal in the gene encoding for the polypep-
tide of interest, which the gene source recognizes and
cleaves with an appropriate peptidase. Since in most
situations, genes of interest are heterologous to the
host in which the gene is to be expressed, such proces-
30 sing occurs imprecisely and in low yield in the expres-
sion host. In this case, while the protein which is
obtained will be identical to the peptide of interest
for almost all of its sequence, it will differ at the
N-terminus which can deleteriously affect physiological
35 activity.

There are, therefore, many reasons why it would be extremely advantageous to prepare DNA sequences, which would encode for the secretion and maturing of the polypeptide product. Furthermore, where sequences can be found for processing, which result in the removal of amino acids superfluous to the polypeptide of interest, the opportunity exists for having a plurality of DNA sequences, either the same or different, in tandem, which may be encoded on a single transcript.

Description of the Prior Art

U.S. Patent No. 4,336,336 describes for prokaryotes the use of a leader sequence coding for a non-cytoplasmic protein normally transported to or beyond the cell surface, resulting in transfer of the fused protein to the periplasmic space. U.S. Patent No. 4,338,397 describes for prokaryotes using a leader sequence which provides for secretion with cleavage of the leader sequence from the polypeptide sequence of interest. U.S. Patent No. 4,338,397, columns 3 and 4, provide for useful definitions, which definitions are incorporated herein by reference.

Kurjan and Herskowitz, Cell (1982) 30:933-943 describes a putative α -factor precursor containing four tandem copies of mature α -factor, describing the sequence and postulating a processing mechanism. Kurjan and Herskowitz, Abstracts of Papers presented at the 1981 Cold Spring Harbor meeting on The Molecular Biology of Yeasts, page 242, in an Abstract entitled, "A Putative α -Factor Precursor Containing Four Tandem Repeats of Mature α -Factor," describe the sequence encoding for the α -factor and spacers between two of such sequences. Blair et al., Abstracts of Papers, ibid, page 243, in an Abstract entitled "Synthesis and Processing of Yeast Pheromones: Identification and Characterization of Mutants That Produce Altered α -

Factors," describe the effect of various mutants on the production of mature α -factor.

SUMMARY OF THE INVENTION

Methods and compositions are provided for
5 producing mature polypeptides. DNA constructs are provided which join the DNA fragments encoding for a yeast leader sequence and processing signal to heterologous genes for secretion and maturation of the polypeptide product. The construct of the DNA encoding for
10 the N-terminal cleavable oligopeptide and the DNA sequence encoding for the mature polypeptide product can be joined to appropriate vectors for introduction into yeast or other cell which recognizes the processing
-- signals for production of the desired polypeptide.
15 Other capabilities may also be introduced into the construct for various purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram indicating the construction of pY α EGF-21.

20 Fig. 2 shows sequences at fusions of hEGF to the vector. a. through e. show the sequences at the N-terminal region of hEGF, which differ among several constructions and f. shows the C-terminal region of hEGF.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

25 In accordance with the subject invention, eukaryotic hosts, particularly yeast are employed for the production of mature polypeptides, where such polypeptides may be harvested from a nutrient medium.
30 The polypeptides are produced by employing a DNA construct encoding for yeast leader and processing signals joined to a polypeptide of interest, which may be a single polypeptide or a plurality of polypeptides separated by processing signals. The resulting

construct encodes for a pre-pro-polypeptide which will contain the signals for secretion of the pre-pro-polypeptide and processing of the polypeptide, either intracellularly or extracellularly to the mature
 5 polypeptide.

The constructs of the subject invention will for example have at least the following formula defining a pro-polypeptide:



10 wherein:

R is CGX or AZZ, the codons coding for lysine and arginine, each of the Rs being the same or different;

r is an integer of from 2 to 4, usually 2 to 3, preferably 2 or 4;

15 X is any of the four nucleotides, T, G, C, or A;

Y is G or C;

y is an integer of at least one and usually not more than 10, more usually not more than four,

20 providing for monomers and multimers;

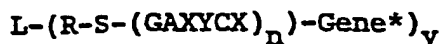
Z is A or G; and

Gene* is a gene other than α -factor, usually foreign to a yeast host, usually a heterologous gene, desirably a plant or mammalian gene;

25 n is 0 or an integer which will generally vary from 1 to 4, usually 2 to 3.

The pro-polypeptide has an N-terminal processing signal for peptidase removal of the amino acids preceding the amino acids encoded for by Gene*.

30 For the most part, the constructs of the subject invention will have at least the following formula:



35 defining a pre-pro-polypeptide, wherein all the symbols except L and S have been defined, S having the same definition as R, there being 1R and 1S, and L is a leader sequence providing for secretion of the pre-pro-polypeptide. While it is feasible to have more

Rs and Ss there will usually be no advantage in the additional amino acids. Any leader sequence may be employed which provides for secretion, leader sequences generally being of about 30 to 120 amino acids, usually
5 about 30 to 100 amino acids, having a hydrophobic region and having a methionine at its N-terminus.

The construct when n is 0 will have the following formula:

$$L-((R)_{r'}-Gene^*)_y$$

10 defining a pre-pro-polypeptide, wherein all the symbols have been defined previously, except r', wherein:

r' is 2 to 4, preferably 2 or 4.

Of particular interest is the leader sequence of α -factor which is described in Kurjan and Hersko-
15 witz, supra, on page 937 or fragments or analogs thereof, which provide for efficient secretion of the desired polypeptides. Furthermore, the DNA sequence indicated in the article, which sequence is incorporated herein by reference, is not essential, any sequence
20 which encodes for the desired oligopeptide being sufficient. Different sequences will be more or less efficiently translated.

While the above formulas are preferred, it should be understood, that with suppressor mutants,
25 other sequences could be provided which would result in the desired function. Normally, suppressor mutants are not as efficient for expression and, therefore, the above indicated sequence or equivalent sequence encoding for the same amino acid sequence is preferred. To the
30 extent that a mutant will express from a different codon the same amino acids which are expressed by the above sequence, then such alternative sequence could be permitted.

The dipeptides which are encoded for by the
35 sequence in the parenthesis will be an acidic amino acid, aspartic or glutamic, preferably glutamic,

followed by a neutral amino acid, alanine and proline, particularly alanine.

- In providing for useful DNA sequences which can be used for cassettes for expression, the following sequence can be conveniently employed:



wherein:

- Tr intends a DNA sequence encoding for the transcriptional regulatory signals, particularly the promoter and such other regulatory signals as operators, activators, cap signal, signals enhancing ribosomal binding, or other sequence involved with transcriptional or translational control. The Tr sequence will generally be at least about 100bp and not more than about 2000bp. Particularly useful is employing the Tr sequence associated with the leader sequence L, so that a DNA fragment can be employed which includes the transcriptional and translational signal sequences associated with the leader sequence endogenous to the host. Alternatively, one may employ other transcriptional and translational signals to provide for enhanced production of the expression product;

d is 0 or 1, being 1 when y is greater than 1;

- n' is a whole number, generally ranging from 0 to 3, more usually being 0 or 2 to 3;

r'' is 1 or 2;

- W intends a terminal deoxyribosyl-3' group, or a DNA sequence which by itself or, when n' is other than 0, in combination with the nucleotides to which it is joined, W defines a restriction site, having either a cohesive end or butt end, wherein W may have from 0 to about 20 nucleotides in the longest chain;

- the remaining symbols having been defined previously.

Of particular interest is the following construct:

$$(\text{Tr})_a - \text{L} - (\text{R-S})_{r''} - (\text{GAXYCK})_{n''} \text{GA} \boxed{\text{AGCT}}$$

wherein:

all of the symbols previously defined have the same definition;

5 a is 0 or 1 intending that the construct may or may not have the transcriptional and translational signals;

the nucleotides indicated in the broken box are intended not to be present but to be capable of
10 addition by adding an HindIII cleaved terminus to provide for the recreation of the sequence encoding for a dipeptide; and

n'' will be 0 to 2, where at least one of the Xs and Ys defines a nucleotide, so that the sequence in
15 the parenthesis is other than the sequence GAAGCT.

The coding sequence of Gene* may be joined to the terminal T, providing that the coding sequence is in frame with the initiation codon and upon processing the first amino acid will be the correct amino acid for
20 the mature polypeptide.

The 3'-terminus of Gene* can be manipulated much more easily and, therefore, it is desirable to provide a construct which allows for insertion of Gene* into a unique restriction site in the construct. Such
25 a construct would provide for a restriction site with insertion of the Gene* into the restriction site to be in frame with the initiation codon. Such a construction can be symbolized as follows:

$$(\text{Tr})_a - \text{L} - (\text{R-S})_{r''} - (\text{GAXYCK})_{n''} - \text{W} - (\text{SC})_b - \text{Te}$$

30 wherein:

those symbols previously defined have the same definition;

SC are stop codons;

Te is a termination sequence balanced with
35 the promoter Tr, and may include other signals, e.g. polyadenylation; and

b is an integer which will generally vary from about 0 to 4, more usually from 0 to 3, it being understood, that Gene* may include its own stop codons.

Illustrative of a sequence having the above
5 formula is where W is the sequence GA and n" is 2.

Of particular interest is where the sequence
encoding the terminal dipeptide is taken together with
W to define a linker or connector, which allows for
recreation of the terminal sequence defining the
10 dipeptide of the processing signal and encodes for the
initial amino acids of Gene*, so that the codons are in
frame with the initiation codon of the leader. The
linker provides for a staggered or butt ended termina-
tion, desirably defining a restriction site in conjunc-
15 tion with the successive sequences of the Gene*. Upon
ligation of the linker with Gene*, the codons of Gene*
will be in frame with the initiation codon of the
leader. In this manner, one can employ a synthetic
sequence which may be joined to a restriction site in
20 the processing signal sequence to recreate the proces-
sing signal, while providing the initial bases of the
Gene* encoding for the N-terminal amino acids. By
employing a synthetic sequence, the synthetic linker
can be a tailored connector having a convenient restric-
25 tion site near the 3'-terminus and the synthetic
connector will then provide for the necessary codons
for the 5'-terminus of the gene.

Alternatively, one could introduce a restric-
tion endonuclease recognition site downstream from the
30 processing signal to allow for cleavage and removal of
superfluous bases to provide for ligation of the Gene*
to the processing signal in frame with the initiation
codon. Thus the first codon would encode for the
N-terminal amino acid of the polypeptide. Where T is
35 the first base of Gene*, one could introduce a restric-
tion site where the recognition sequence is downstream
from the cleavage site. For example, a Sau3A recogni-

tion sequence could be introduced immediately after the processing signal, which would allow for cleavage and linking of the Gene* with its initial codon in frame with the leader initiation codon. With restriction
5 endonucleases which have the recognition sequence distal and downstream from the cleavage site e.g. HgaI, W could define such sequence which could include a portion of the processing signal sequences. Other constructions can also be employed, employing such
10 techniques as primer repair and in vitro mutagenesis to provide for the convenient insertion of Gene* into the construct by introducing an appropriate restriction site.

The construct provides a portable sequence
15 for insertion into vectors, which provide the desired replication system. As already indicated, in some instances, it may be desirable to replace the wild type promoter associated with the leader sequence with a different promoter. In yeast, promoters involved with
20 enzymes in the glycolytic pathway can provide for high rates of transcription. These promoters are associated with such enzymes as phosphoglucoisomerase, phosphofructokinase, phosphotriose isomerase, phosphoglucomutase, enolase, pyruvic kinase, glyceraldehyde-3-
25 phosphate dehydrogenase, and alcohol dehydrogenase. These promoters may be inserted upstream from the leader sequence. The 5'-flanking region to the leader sequence may be retained or replaced with the 3'-sequence of the alternative promoter. Vectors can be
30 prepared and have been reported which include promoters having convenient restriction sites downstream from the promoter for insertion of such constructs as described above.

The final construct will be an episomal
35 element capable of stable maintenance in a host, particularly a fungal host such as yeast. The construct will include one or more replication systems, desirably

two replication systems, allowing for maintenance in the expression host and cloning in a prokaryote. In addition, one or more markers for selection will be included, which will allow for selective pressure for maintenance of the episomal element in the host. Furthermore, the episomal element may be a high or low copy number, the copy number generally ranging from about 1 to 200. With high copy number episomal elements, there will generally be at least 10, preferably at least 20, and usually not exceeding about 150, more usually not exceeding about 100 copy number. Depending upon the Gene*, either high or low copy numbers may be desirable, depending upon the effect of the episomal element on the host. Where the presence of the expression product of the episomal element may have a deleterious effect on the viability of the host, a low copy number may be indicated.

Various hosts may be employed, particularly mutants having desired properties. It should be appreciated that depending upon the rate of production of the expression product of the construct, the processing enzyme may or may not be adequate for processing at that level of production. Therefore, a mutant having enhanced production of the processing enzyme may be indicated or enhanced production of the enzyme may be provided by means of an episomal element. Generally, the production of the enzyme should be of a lower order than the production of the desired expression product.

Where one is using α -factor for secretion and processing, it would be appropriate to provide for enhanced production of the processing enzyme Dipeptidyl Amino Peptidase A, which appears to be the expression product of STE13. This enzyme appears to be specific for X-Ala- and X-Pro-sequences, where X in this instance intends an amino acid, particularly, the dicarboxylic acid amino acids.

Alternatively, there may be situations where intracellular processing is not desired. In this situation, it would be useful to have a stel3 mutant, where secretion occurs, but the product is not processed. In this manner, the product may be subsequently processed in vitro.

Host mutants which provide for controlled regulation of expression may be employed to advantage. For example, with the constructions of the subject invention where a fused protein is expressed, the transformants have slow growth which appears to be a result of toxicity of the fused protein. Thus, by inhibiting expression during growth, the host may be grown to high density before changing the conditions to permissive conditions for expression.

A temperature-sensitive sir mutant may be employed to achieve regulated expression. Mutation in any of the SIR genes results in a non-mating phenotype due to in situ expression of the normally silent MATa and MAT α sequences present at the HML and HMR loci.

Furthermore, as already indicated, the Gene* may have a plurality of sequences in tandem, either the same or different sequences, with intervening processing signals. In this manner, the product may be processed in whole or in part, with the result that one will obtain the various sequences either by themselves or in tandem for subsequent processing. In many situations, it may be desirable to provide for different sequences, where each of the sequences is a subunit of a particular protein product.

The Gene* may encode for any type of polypeptide of interest. The polypeptide may be as small as an oligopeptide of 8 amino acids or may be 100,000 daltons or higher. Usually, single chains will be less than about 300,000 daltons, more usually less than about 150,000 daltons. Of particular interest are polypeptides of from about 5,000 to 150,000 daltons,

more particularly of about 5,000 to 100,000 daltons. Illustrative polypeptides of interest include hormones and factors, such as growth hormone, somatomedins epidermal growth factor, the endocrine secretions, such as luteinizing hormone, thyroid stimulating hormone, oxytocin, insulin, vasopressin, renin, calcitonin, follicle stimulating hormone, prolactin, etc.; hematopoietic factors, e.g. erythropoietin, colony stimulating factor, etc.; lymphokines; globins; globulins, e.g. immunoglobulins; albumins; interferons, such as α , β and γ ; repressors; enzymes; endorphins e.g. β -endorphin, enkephalin, dynorphin, etc.

Having prepared the episomal elements containing the constructs of this invention, one may then introduce such element into an appropriate host. The manner of introduction is conventional, there being a wide variety of ways to introduce DNA into a host. Conveniently, spheroplasts are prepared employing the procedure of, for example, Hinnen et al., PNAS USA (1978) 75:1919-1933 or Stinchcomb et al., EP 0 045 573 A2. The transformants may then be grown in an appropriate nutrient medium and where appropriate, maintaining selective pressure on the transformants. Where expression is inducible, one can allow for growth of the yeast to high density and then induce expression. In those situations, where a substantial proportion of the product may be retained in the periplasmic space, one can release the product by treating the yeast cells with an enzyme such as zymolase or lyticase.

The product may be harvested by any convenient means, purifying the protein by chromatography, electrophoresis, dialysis, solvent-solvent extraction, etc.

In accordance with the subject invention, one can provide for secretion of a wide variety of polypeptides, so as to greatly enhance product yield, simplify purification, minimize degradation of the desired

product, and simplify processing, equipment, and engineering requirements. Furthermore, utilization of nutrients based on productivity can be greatly enhanced, so that more economical and more efficient production of polypeptides may be achieved. Also, the use of yeast has many advantages in avoiding enterotoxins, which may be present with prokaryotes, and employing known techniques, which have been developed for yeast over long periods of time, which techniques include isolation of yeast products.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

A synthetic sequence for human epidermal growth factor (EGF) based on the amino acid sequence of EGF reported by H. Gregory and B.M. Preston Int. J. Peptide Protein Res. 9, 107-118 (1977) was prepared, which had the following sequence.

5' AACTCCGACTCCGAATGTCCATTGTCCACGACGGTTACTGTTGCACGACGGTGTGTTGT
3' TTGAGGCTGAGGCTTACAGGTAACAGGGTGCTGCCAATGACAAACGTGCTGCCACAACAA

ATGTACATCGAAGCTTTGGACAAGTACGCTTGTAAGTGTGTTGTTGGTTACATCGGTGAA
TACATGTAGCTTCGAAACCTGTTTCATGCCGAACATTGACACAACAACCAATGTAGCCACTT

AGATGTCAATACAGAGACTTGAAGTGGTGGGAATTGAGATGA
TCTACAGTTATGTCTCTGAACTTCACCACCCTTAAGTCTACT,

where 5' indicates the promoter proximal end of the sequence. The sequence was inserted into the EcoRI site of pBR328 to produce a plasmid p328EGF-1 and cloned.

Approximately 30µg of p328EGF-1 was digested with EcoRI and approximately 1µg of the expected 190 base pair EcoRI fragment was isolated. This was followed by digestion with the restriction enzyme HgaI.

Two synthetic oligonucleotide connectors HindIII-HgaI and HgaI-SalI were then ligated to the 159 base pair HgaI fragment. The HgaI-HindIII linker had the following sequence:

5 AGCTGAAGCT
CTTCGATTGAG

This linker restores the α -factor processing signals interrupted by the HindIII digestion and joins the HgaI end at the 5'-end of the EGF gene to the HindIII end of pAB112.

The HgaI-SalI linker had the following sequence:

15 TGAGATGATAAG
ACTATTGAGCT
This linker has two stop codons and joins the HgaI end at the 3'-end of the EGF gene to the SalI end of pAB112.

The resulting 181 base pair fragment was purified by preparative gel electrophoresis and ligated to 100ng of pAB112 which had been previously completely digested with the enzymes HindIII and SalI. Surprisingly, a deletion occurred where the codon for the 3rd and 4th amino acids of EGF, asp and ser, were deleted, with the remainder of the EGF being retained.

25 pAB112 is a plasmid containing a 1.75kb EcoRI fragment with the yeast α -factor gene cloned in the EcoRI site of pBR322 in which the HindIII and SalI sites had been deleted (pAB11). pAB112 was derived from plasmid pAB101 which contains the yeast α -factor gene as a partial Sau3A fragment cloned in the BamHI site of plasmid YEp24. pAB101 was obtained by screening a yeast genomic library in YEp24 using a synthetic 20-mer oligonucleotide probe (3'-GGCCGGTTGGTTACATGATT-5') homologous to the published α -factor coding region
35 (Kurjan and Herskowitz, Abstracts 1981 Cold Spring Harbor meeting on the Molecular Biology of Yeasts, page 242).

The resulting mixture was used to transform E. coli HB101 cells and plasmid pAB201 obtained.

Plasmid pAB201 (5µg) was digested to completion with the enzyme EcoRI and the resulting fragments were:

- 5 a) filled in with DNA polymerase I Klenow fragment;
- b) ligated to an excess of BamHI linkers; and
- c) digested with BamHI. The 1.75kbp EcoRI fragment was isolated by preparative gel electrophoresis and approximately 100ng of the fragment was ligated to
- 10 100ng of pC1/1, which had been previously digested to completion with the restriction enzyme BamHI and treated with alkaline phosphatase.

- Plasmid pC1/1 is a derivative of pJDB219, Beggs, Nature (1978) 275:104, in which the region
- 15 corresponding to bacterial plasmid pMB9 in pJDB219 has been replaced by pBR322 in pC1/1. This mixture was used to transform E. coli HB101 cells. Transformants were selected by ampicillin resistance and their plasmids analyzed by restriction endonucleases. DNA
 - 20 from one selected clone (pYEGF-8) was prepared and used to transform yeast AB103 cells. Transformants were selected by their leu⁺ phenotype.

- Fifty milliliter cultures of yeast strain AB103 (α, pep 4-3, leu 2-3, leu 2-112, ura 3-52, his
- 25 4-580) transformed with plasmid pYEGF-8 (deposited at the American Type Culture Collection on 5th January 1983 and given ATCC Accession no. 20658) were grown at 30° in -leu medium to saturation (optical density at 600nm of 5) and left shaking at 30° for an additional
 - 30 12 hr period. Cell supernatants were collected by centrifugation and analyzed for the presence of human EGF using the fibroblast receptor competition binding assay. The assay of EGF is based on the ability of both mouse and human EGF to compete with ¹²⁵I-labeled
 - 35 mouse EGF for binding sites on human foreskin fibroblasts. Standard curves can be obtained by measuring the effects of increasing quantities of EGF on the binding of a standard amount of ¹²⁵I-labeled mouse EGF. Under these conditions 2 to 20 ng of EGF are readily

measurable. Details on the binding of ^{125}I -labeled epidermal growth factor to human fibroblasts have been described by Carpenter et al., J. Biol. Chem. 250, 4297 (1975). Using this assay it is found that the culture
5 medium contains 7 \pm 1mg of human EGF per liter.

For further characterization, human EGF present in the supernatant was purified by absorption to the ion-exchange resin Biorex-70 and elution with HCl 10mM in 80% ethanol. After evaporation of the HCl
10 and ethanol the EGF was solubilized in water. This material migrates as a single major protein of MW approx. 6,000 in 17.5% SDS gels, roughly the same as authentic mouse EGF (MW~6,000). This indicates that the α -factor leader sequence has been properly excised
15 during the secretion process. Analysis by high resolution liquid chromatography (microbondapak C18, Waters column) indicates that the product migrates with a retention time similar to an authentic mouse EGF standard. However, protein sequencing by Edman degradation showed that the N-terminus retained the glu-ala
20 sequence.

A number of other constructions were prepared using different constructions for joining hEGF to the α -factor secretory leader sequence, providing for
25 different processing signals and site mutagenesis. In Fig. 2 a. through e. show the sequence of the fusions at the N-terminal region of hEGF, which sequence differ among several constructions. f. shows the sequences at the C-terminal region of hEGF, which is the same for all
30 constructions. Synthetic oligonucleotide linkers used in these constructions are boxed.

These fusions were made as follows. Construction (a) was made as described above. Construction (b) was made in a similar way except that linker 2 was used
35 instead of linker 1. Linker 2 modifies the α -factor processing signal by inserting an additional processing

site (ser-leu-asp-lys-arg) immediately preceding the hEGF gene. The resulting yeast plasmid is named pY α EGF-22. Construction (c), in which the dipeptidyl aminopeptidase maturation site (glu-ala) has been removed, was obtained by in vitro mutagenesis of construction (a). A PstI-SalI fragment containing the α -factor leader-hEGF fusion was cloned in phage M13 and isolated in a single-stranded form. A synthetic 31-mer of sequence 5'-TCTTTGGATAAAAGAACTCCGACTCCCG-3' was synthesized and 70 picomoles were used as a primer for the synthesis of the second strand from 1 picomole of the above template by the Klenow fragment of DNA polymerase. After fill-in and ligation at 14° for 18 hrs, the mixture was treated with S₁ nuclease (5 units for 15 min) and used to transfect E. coli JM101 cells. Bacteriophage containing DNA sequences in which the region coding for (glu-ala) was removed were located by filter plaque hybridization using the ³²P-labeled primer as probe. RF DNA from positive plaques was isolated, digested with PstI and SalI and the resulting fragment inserted in pAB114 which had been previously digested to completion with SalI and partially with PstI and treated with alkaline phosphatase.

The plasmid pAB114 was derived as follows:

25 plasmid pAB112 was digested to completion with HindIII and then religated at low (4 μ g/ml) DNA concentration and plasmid pAB113 was obtained in which three 63bp HindIII fragments have been deleted from the α -factor structural gene, leaving only a single copy of mature

30 α -factor coding region. A BamHI site was added to plasmid pAB11 by cleavage with EcoRI, filling in of the overhanging ends by the Klenow fragment of DNA polymerase, ligation of BamHI linkers, cleavage with BamHI and religation to obtain pAB12. Plasmid pAB13

35 was digested with EcoRI, the overhanging ends filled in, and ligated to BamHI linkers. After digestion with BamHI the 1500bp fragment was gel-purified and ligated

to pAB12 which had been digested with BamHI and treated with alkaline phosphatase. Plasmid pAB114, which contains a 1500bp BamHI fragment carrying the g-factor gene, was obtained. The resulting plasmid (pAB114
5 containing the above described construct) is then digested with BamHI and ligated into plasmid pCl1/1.

The resulting yeast plasmid is named pY α EGF-23 and was deposited at the American Type Culture Collection on 12th August 1983 under ATCC Accession no. 40079.

10 Construction (d), in which a new KpnI site was generated, was made as described for construction (c) except that the 36-mer oligonucleotide primer of sequence 5'-GGGTACCTTTGGATAAAAGAAACTCCGACTCCGAAT-3' was used. The resulting yeast plasmid is named pY α EGF-24.

15 Construction (e) was derived by digestion of the plasmid containing construction (d) with KpnI and SalI instead of linker 1 and 2. The resulting yeast plasmid is named pY α EGF-25.

Yeast cells transformed with pY α EGF-22 were
20 grown in 15 ml cultures. At the indicated densities or times, cultures were centrifuged and the supernatants saved and kept on ice. The cell pellets were washed in lysis buffer (0.1 Triton X-100, 10mM NaHPO₄ pH 7.5) and broken by vortexing (5min in 1min intervals with
25 cooling on ice in between) in one volume of lysis buffer and one volume of glass beads. After centrifugation, the supernatants were collected and kept on ice. The amount of hEGF in the culture medium and cell
30 binding competition assay. Standard curves were obtained by measuring the effects of increasing quantities of mouse EGF on the binding of a standard amount ¹²⁵I-labeled mouse EGF.

Proteins were concentrated from the culture
35 media by absorption on Bio-Rex 70 resin and elution with 0.01 HCl in 80% ethanol and purified by high performance liquid chromatography (HPLC) on a reverse phase C18 column. The column was eluted at a flow rate of 4ml/min with a linear gradient of 5% to 80% aceto-

nitrile containing 0.2% trifluoroacetic acid in 60min. Proteins (200-800 picomoles) were sequenced at the amino-terminal end by the Edman degradation method using a gas-phase protein sequencer Applied Biosystems model 470A. The normal PROTFA program was used for all the analyses. Dithiothreitol was added to S2 (ethyl acetate: 20mg/liter) and S3 (butyl chloride: 10mg/liter) immediately before use. All samples were treated with 1N HCl in methanol at 40° for 15min to convert PTH-aspartic acid and PTH-glutamic acid to their methyl esters. All PTH-amino acid identifications were performed by reference to retention times on a IBM CN HPLC column using a known mixture of PTH-amino acids as standards.

Secretion from pYαEGF-22 gave a 4:1 mole ratio of native N-terminus hEGF to glu-ala terminated hEGF, while secretion from pYαEGF-23-25 gave only native N-terminated hEGF. Yields of hEGF ranged from 5 to 8µg/ml measured either as protein or in a receptor binding assay.

The strain JRY188 (MAT sir3-8 leu2-3 leu2-112 trp1 ura3 his4 rme) was transformed with pYαEGF-21 and leucine prototrophs selected at 37°. Saturated cultures were then diluted 1/100 in fresh medium and grown in leucine selective medium at permissive (24°) and non-permissive (36°) temperatures and culture supernatants were assayed for the presence of hEGF as described above. The results are shown in the following table.

Regulated synthesis and secretion of hEGF in transformed yeast sir3 temperature-sensitive mutants.

5	Temperature	Transformant	O.D.650	hEGF(μ g/ml)
	36°	3a	3.5 5.4	0.010 0.026
10		3b	3.6 6.4	0.020 0.024
	24°	3a	0.4 1.3 2.1 4.0	34 145 1075 3250
15		3b	0.4 1.4 2.2 4.2	32 210 1935 4600
20				

These results indicate that the hybrid α -factor/EGF gene is being expressed under mating type regulation, even though it is present on a high copy number plasmid.

25 In accordance with the subject invention, novel constructs are provided which may be inserted into vectors to provide for expression of polypeptides having an N-terminal leader sequence and one or more processing signals to provide for secretion of the

30 polypeptide as well as processing to result in a mature polypeptide product free of superfluous amino acids. Thus, one can obtain a polypeptide having the identical sequence to a naturally occurring polypeptide. In addition, because the polypeptide can be produced in

35 yeast, glycosylation can occur, so that products can be obtained which are identical to the naturally occurring

products. Furthermore, because the product is secreted, greatly enhanced yields can be obtained based on cell population and processing and purification are greatly simplified. In addition, employing mutant hosts, 5 expression can be regulated to be turned off or on, as desired.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it 10 will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

CLAIMS

1. A DNA construct encoding a pre-pro-polypeptide, said DNA construct encoding pre-pro-polypeptide comprising a yeast leader sequence, processing signals
5 for processing the pre-pro-polypeptide to a mature polypeptide and a gene encoding a polypeptide other than the wild type gene associated with said leader sequence.

2. A DNA construct according to Claim 1, including at the 5' end of the sequence a yeast promoter and wherein said gene is heterologous to said
10 yeast host.

3. A DNA construct according to Claim 2, wherein said yeast promoter is the α -factor promoter and said yeast leader is a leader sequence encoding for
15 at least a major portion of the α -factor leader and is capable of providing for secretion.

4. A DNA construct according to Claim 2, wherein said gene is a mammalian gene.

5. A DNA construct comprising a sequence of
20 the following formula:



wherein:

L is a leader sequence recognized by yeast for secretion;

25 R is a codon coding for arginine or lysine;

r is an integer of from 2 to 4;

X is any nucleotide;

Y is guanosine or cytosine;

y is an integer of from about 1 to 10;

30 Gene* is a gene foreign to yeast; and

n is 0 or 1 to 4.

6. A DNA construct according to Claim 5, wherein n is 0 to 4 and the nucleotides of said Gene* proximal to R at least in part define a recognition site for a restriction endonuclease.

5 7. A DNA construct according to Claim 6, wherein said leader sequence is the α -factor leader sequence.

8. A DNA construct according to Claim 7, wherein n is 0.

10 9. A DNA construct of the formula:
Tr-L-(R-S-(GAXYCX)_n'-W-(Gene*)_d)_y

wherein:

Tr is a sequence having transcriptional and translational regulatory signals for initiation and processing of transcription and translation, wherein
15 said regulatory signals are recognized by yeast;

L is a leader sequence for secretion by yeast;

R and S are codons expressing arginine and
20 lysine;

X is any nucleotide;

Y is cytosine or guanosine;

y is an integer of from 1 to 4;

n' is a whole number of from 0 to 4;

25 W is a deoxyribosyl-3' group or when n' is other than 0, one or more nucleotides which by themselves or together with the hexanucleotide in the parenthesis define a restriction site;

Gene* either by itself or taken together with
30 W defines a polypeptide sequence foreign to yeast; and
d is 0 or 1, being 1, when y is greater than

1.

10. A DNA construct according to Claim 9, wherein Tr is a sequence defining the regulatory signals for α -factor, d is 1 and Gene* and W are taken together to define a polypeptide foreign to yeast.

5 11. A DNA construct according to Claim 9 wherein n' is 0.

12. A DNA construct according to Claim 11, wherein said polypeptide product is a mammalian polypeptide.

10 13. A DNA construct comprising a sequence of the formula:

$(Tr)_a-L-R-S-(GAXYCX)_{n''}GA\boxed{\overline{AGCT}}$

wherein:

15 Tr is a sequence defining transcriptional and translational regulatory signals for initiation and processing of transcription and translation recognized by yeast;

a is 0 or 1;

L is a leader sequence recognized by yeast;

20 R and S are codons encoding for lysine and arginine;

X is any nucleotide;

Y is cytosine or guanosine;

n'' is 2 to 4;

25 the nucleotides in the broken box indicate the nucleotides which are complementary to the overhang of the non-coding chain to define a HindIII restriction site.

14. A DNA construct according to Claim 13, where Tr is a sequence defining the transcriptional and translational regulatory signals of α -factor.

15. An expression episomal element comprising a replication system for providing stable maintenance in yeast and a sequence of the formula:

$$\text{Tr-L-(R)}_r\text{-(GAXYCX)}_{n'}\text{-W-Te}$$

wherein:

Tr is a sequence defining transcriptional and translational regulatory signals for initiation and processing of transcription and translation in yeast;

L is a leader sequence recognized by yeast for secretion;

R is a codon defining arginine or lysine;

r' is a whole number in the range of 2 to 4;

X is any nucleotide;

Y is cytosine or guanosine;

n' is a whole number in the range of 0 to 4;

W is a nucleotide sequence of at least 1 nucleotide, which by itself or when n' is other than 0, in conjunction with nucleotides in the parenthesis defines a restriction site;

Te is a sequence defining a terminator balanced with said transcriptional initiator sequence.

16. An expression episomal element according to Claim 15 wherein Tr is derived from α -factor and n' is 2 to 3.

17. An expression episomal element according to Claim 14, wherein Tr is derived from α -factor and n' is 0.

18. An episomal expression vector according to Claim 17, having a gene foreign to yeast intermediate

R and Te and in reading frame with the initiation codon of L.

19. An episomal expression element according to Claim 18, wherein said gene is a mammalian gene.

5 20. An episomal element according to Claim 19, wherein said mammalian gene is human epidermal growth factor.

10 21. An episomal expression vector according to Claim 16, having a gene foreign to yeast intermediate the nucleotides in the parentheses and Te and in reading frame with the initiation codon of L.

15 22. A method for producing a polypeptide foreign to yeast and having such polypeptide secreted into the culture medium, said method comprising:
growing yeast containing an episomal expression elements according to Claim 16, whereby the encoding sequences are expressed to produce a pre-pro-polypeptide; and
20 said pre-pro-polypeptide is at least partially processed and secreted.

23. An episomal expression vector according to Claim 17, having a gene foreign to yeast intermediate the nucleotides in the parentheses and Te and in reading frame with the initiation codon of L.

25 24. A method for producing a polypeptide foreign to yeast and having such polypeptide secreted into the culture medium, said method comprising:
growing yeast mutants containing an episomal expression element according to Claim 16, wherein said
30 mutant permits external regulation of expression,

whereby the encoding sequences are expressed to produce a pre-pro-polypeptide under permissive conditions; and said pre-pro-polypeptide is at least partially processed and secreted.

5 25. A method according to Claim 24, wherein said mutant yeast is a temperature-sensitive sir mutant.

10 26. A method for producing a polypeptide foreign to yeast and having such polypeptide secreted into the culture medium, said method comprising:
 growing yeast mutants containing an episomal expression element according to Claim 17, wherein said mutant permits external regulation of expression, whereby the encoding sequences are expressed to produce
15 a pre-pro-polypeptide under permissive conditions; and
 said pre-pro-polypeptide is at least partially processed and secreted.

20 27. A method according to Claim 26, wherein said mutant yeast is a temperature-sensitive sir mutant.

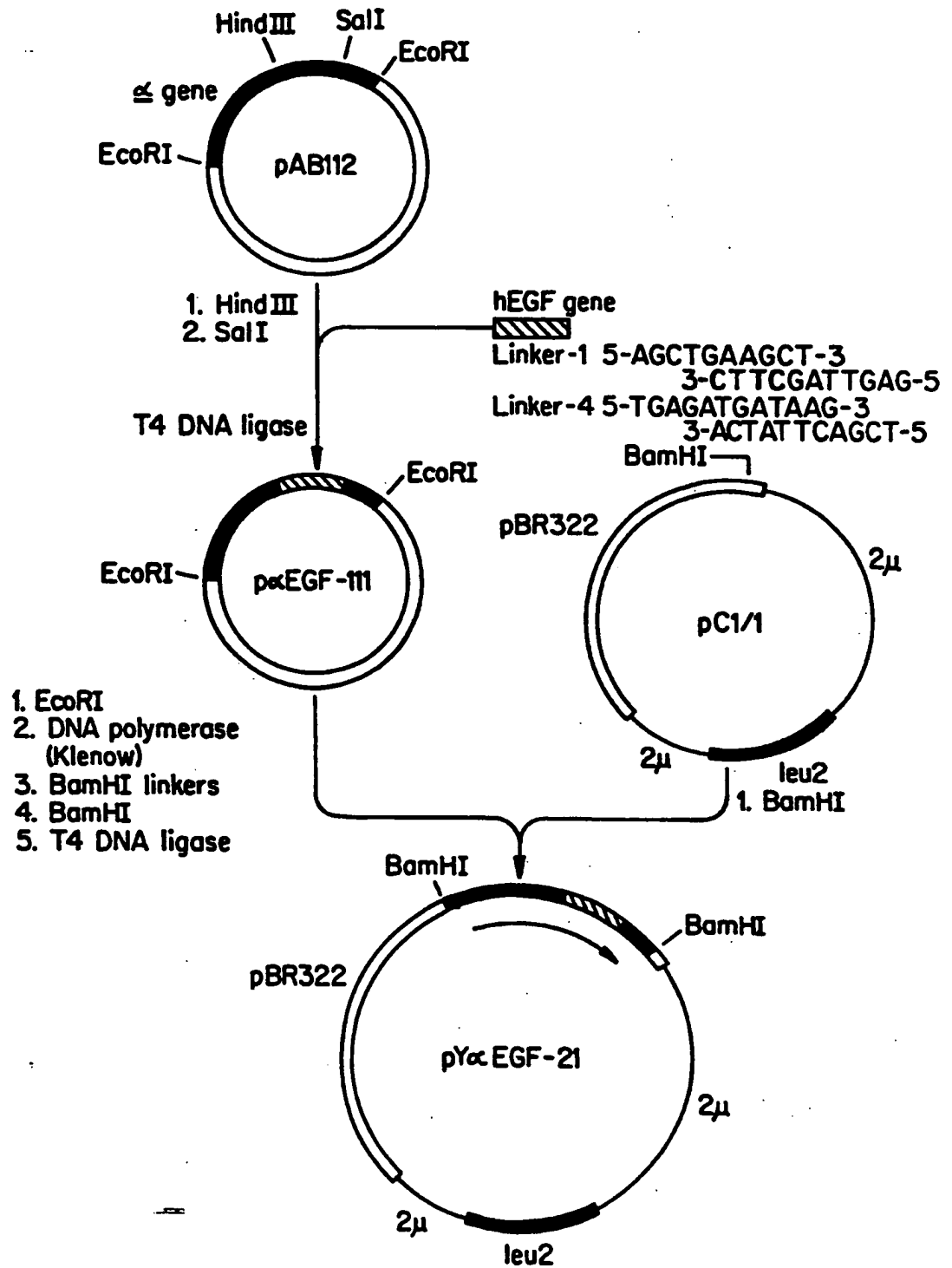


FIG. 1.

80 85 90 5
Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Glu Ala Asn Ser Asp Ser Glu
a. GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT GAA GCT AAC TCC GAC TCC GAA
CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA CTT CGA TTG AGG CTG AGG CTT
Linker-1
80 85 90 5
Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
b. GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
Linker-2
80 85 5
Gly Val Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
c. GGG GTA TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
CCC CAT AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
80 85 5
Gly Val Pro Leu Asp Lys Arg Asn Ser Asp Ser Glu
KpnI
d. GGG GTA CCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
CCC CAT GGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
80 85 5
Gly Val Gin Leu Asp Lys Arg Asn Ser Asp Ser Glu
PvuII Linker-3
e. GGG GTA GAG CTG GAT AAA AGA AAC TCC GAC TCC GAA
CCC CAT GTC GAC CTA TTT TCT TTG AGG CTG AGG CTT
50 53
Trp Trp Glu Leu Arg
Linker-4
f. TGG TGG GAA TTG AGA TGA TAA GTC GAC CGA TG
ACC ACC CTT AAC TCT ACT ATT CAG CTG GCT AC

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FIG. 2.



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EUROPEAN SEARCH REPORT

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Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 83306507.1
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 7)
X,P	EP - A2 - 0 088 632 (GENENTECH) * Claims 1,5,15; page 5, lines 21-28; page 6, lines 4-19 * --	1,2,4, 22,24, 26	C 12 N 15/00 C 07 H 21/04 C 12 P 21/00 //C 12 R 1/645
A	EP - A1 - 0 060 057 (BOARD OF REGENTS UNIVERSITY OF WASHINGTON) * Claims 1,6,7 * --	1,2	
A	GB - A - 2 100 737 (CELLTECH) * Abstract; claim 4 * --	1	
A,P	EP - A2 - 0 073 635 (KINGSMAN) * Claim 1 * --	1,2	
A,P	GB - A - 2 104 902 (GENENTECH) * Claim 1 * --	1,2,4	TECHNICAL FIELDS SEARCHED (Int. Cl. 7)
A,D	CELL, vol. 30, no. 3, October 1982, Cambridge, Massachusetts and London J. KURJAN, I. HERSKOWITZ "Structure of a Yeast Pheromone Gene (MF _{ec}): A Putative α -Factor Precursor Contains Four Tandem Copies of Mature α -Factor" pages 933-943 * Page 933 (SUMMARY) * --	3	C 12 N C 12 P C 07 H
A,D	US - A - 4 338 397 (GILBERT) * Claims 1,7,8,12,16 * ----	1,2	
The present search report has been drawn up for all claims			
Place of search VIENNA		Date of completion of the search 13-04-1984	Examiner FARNIOK
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	



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| EP-A- 0 060 057 | EP-A- 0 073 635 |
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| GB-A- 2 104 902 | US-A- 4 338 397 |

The Molec. Biology of Yeast, 11-16.08.1981, p. 242 and 243;

Nucleic Acids Research, Vo. 10, No. 15, 1982, pp. 4467 - 4482;

EPO publication 46 039 (G. D. Searle)

CELL, Vol. 30, n 3, October 1982, Cambridge, Massachusetts and London. J. Kurjan, I. Her-

skowitz "Structure of a Yeast Pheromone Gene (MFx) : A putative x-factor precursor contains four tandem copies of mature x-factor", pages 933-943

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Description**BACKGROUND OF THE INVENTION**5 **Field of the Invention**

Hybrid DNA technology has revolutionized the ability to produce polypeptides of an infinite variety of compositions. Since living forms are composed of proteins and employ proteins for regulation, the ability to duplicate these proteins at will offers unique opportunities for investigating the manner in which these
10 proteins function and the use of such proteins, fragments of such proteins, or analogs in therapy and diagnosis.

There have been numerous advances in improving the rate and amount of protein produced by a cell. Most of these advances have been associated with higher copy numbers, more efficient promoters, and means for reducing the amount of degradation of the desired product. It is evident that it would be
15 extremely desirable to be able to secrete polypeptides of interest, where such polypeptides are the product of interest.

Furthermore, in many situations, the polypeptide of interest does not have an initial methionine amino acid. This is usually a result of there being a processing signal in the gene encoding for the polypeptide of interest, which the gene source recognizes and cleaves with an appropriate peptidase. Since in most
20 situations, genes of interest are heterologous to the host in which the gene is to be expressed, such processing occurs imprecisely and in low yield in the expression host. In this case, while the protein which is obtained will be identical to the peptide of interest for almost all of its sequence, it will differ at the N-terminus which can deleteriously affect physiological activity.

There are, therefore, many reasons why it would be extremely advantageous to prepare DNA
25 sequences, which would encode for the secretion and maturing of the polypeptide product. Furthermore, where sequences can be found for processing, which result in the removal of amino acids superfluous to the polypeptide of interest, the opportunity exists for having a plurality of DNA sequences, either the same or different, in tandem, which may be encoded on a single transcript.

30 **Description of the Prior Art**

U.S. Patent No. 4,336,336 describes for prokaryotes the use of a leader sequence coding for a non-cytoplasmic protein normally transported to or beyond the cell surface, resulting in transfer of the fused protein to the periplasmic space. U.S. Patent No. 4,338,397 describes for prokaryotes using a leader
35 sequence which provides for secretion with cleavage of the leader sequence from the polypeptide sequence of interest. U.S. Patent No. 4,338,397, columns 3 and 4, provide for useful definitions, which definitions are incorporated herein by reference.

Kurjan and Herskowitz, Cell (1982) 30:933-943 describes a putative α -factor precursor containing four tandem copies of mature α -factor, describing the sequence and postulating a processing mechanism.
40 Kurjan and Herskowitz, Abstracts of Papers presented at the 1981 Cold Spring Harbor meeting on The Molecular Biology of Yeasts, page 242, in an Abstract entitled, "A Putative α -Factor Precursor Containing Four Tandem Repeats of Mature α -Factor," describe the sequence encoding for the α -factor and spacers between two of such sequences. Blair et al., Abstracts of Papers, *ibid*, page 243, in an Abstract entitled "Synthesis and Processing of Yeast Pheromones: Identification and Characterization of Mutants That
45 Produce Altered α -Factors," describe the effect of various mutants on the production of mature α -factor.

SUMMARY OF THE INVENTION

The subject matter of the invention is defined in the claims.

50 Methods and compositions are provided for producing mature polypeptides. DNA constructs are provided which join the DNA fragments encoding for a yeast leader sequence and processing signal to heterologous genes for secretion and maturation of the polypeptide product. The construct of the DNA encoding for the N-terminal cleavable α -factor leader (or fragments or analogs thereof) and the DNA sequence encoding for the mature polypeptide product can be joined to appropriate vectors for introduction
55 into yeast or other cell which recognizes the processing signals for production of the desired polypeptide. Other capabilities may also be introduced into the construct for various purposes.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a flow diagram indicating the construction of pYαEGF-21.

Fig. 2 shows sequences at fusions of hEGF to the vector. a. through e. show the sequences at the N-terminal region of hEGF, which differ among several constructions and f. shows the C-terminal region of hEGF.

5

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, eukaryotic hosts, particularly yeast are employed for the production of mature polypeptides, where such polypeptides may be harvested from a nutrient medium.

10 The polypeptides are produced by employing a DNA construct encoding for an alpha-factor leader (or fragments or analogs thereof) and processing signals joined to a polypeptide of interest, which may be a single polypeptide or a plurality of polypeptides separated by processing signals. The resulting construct encodes for a pre-pro-polypeptide which will contain the signals for secretion of the pre-pro-polypeptide and processing of the polypeptide, either intracellularly or extracellularly to the mature polypeptide.

15 According to a preferred embodiment of the invention, there is provided a DNA construct comprising a sequence comprising the formula:

5'-Tr-L-SP-Gene*-Te-3'

20 wherein:

Tr is a yeast promoter sequence;

L encodes at least a yeast alpha-factor leader sequence fragment that provides for secretion;

Sp is a spacer sequence encoding processing signals for processing the precursor polypeptide encoded by L-Sp-Ge-ne* into the polypeptide encoded by Gene*;

25 Gene* encodes a polypeptide foreign to yeast; and Te is a transcription termination sequence balanced with Tr.

Furthermore, a DNA construct of the above formula is preferred wherein Sp contains or is composed of the sequence 5'-R₁-R₂-3' immediately adjacent to the sequence Gene*, R₁ being a codon for lysine or arginine, R₂ being a codon for arginine but does not encode a processing signal for dipeptidylaminopeptidase A.

30 The constructs of the subject invention will for example have at least the following formula defining a propolypeptide:

$((R)_r-(GAXYCX)_n-Gene^*)_y$

35 wherein:

R is CGX or AZZ, the codons coding for lysine and arginine, each of the Rs being the same or different;

r is an integer of from 2 to 4, usually 2 to 3, preferably 2 or 4;

X is any of the four nucleotides, T, G, C, or A;

40 Y is G or C;

y is an integer of at least one and usually not more than 10, more usually not more than four, providing for monomers and multimers;

Z is A or G; and

45 Gene* is a gene other than α-factor, usually foreign to a yeast host, usually a heterologous gene, desirably a plant or mammalian gene;

n is 0 or an integer which will generally vary from 1 to 4, usually 2 to 3.

The pro-polypeptide has an N-terminal processing signal for peptidase removal of the amino acids preceding the amino acids encoded for by Gene*.

For the most part, the constructs of the subject invention will have at least the following formula:

50

$L-(R-S-(GAXYCX)_n-Gene^*)_y$

55 defining a pre-pro-polypeptide, wherein all the symbols except L and S have been defined, S having the same definition as R, there being 1R and 1S, and L is an alpha-factor leader sequence (or fragment or analog thereof) providing for secretion of the pre-pro-polypeptide. While it is feasible to have more Rs and Ss there will usually be no advantage in the additional amino acids. Any alpha-factor leader sequence (or fragment or analog thereof) may be employed which provides for secretion, leader sequences generally being of about 30 to 120 amino acids, usually about 30 to 100 amino acids, having a hydrophobic region

and having a methionine at its N-terminus.

The construct when n is 0 will have the following formula:

$L-((R)_{r'}-Gene)_y$

5

defining a pre-pro-polypeptide, wherein all the symbols have been defined previously, except r' , wherein:
 r' is 2 to 4, preferably 2 or 4.

Of particular interest is the leader sequence of α -factor which is described in Kurjan and Herskowitz, supra, on page 937 or fragments or analogs thereof, which provide for efficient secretion of the desired polypeptides. Furthermore, the DNA sequence indicated in the article, which sequence is incorporated herein by reference, is not essential, any sequence which encodes for the desired oligopeptide being sufficient. Different sequences will be more or less efficiently translated.

While the above formulas are preferred, it should be understood, that with suppressor mutants, other sequences could be provided which would result in the desired function. Normally, suppressor mutants are not as efficient for expression and, therefore, the above indicated sequence or equivalent sequence encoding for the same amino acid sequence is preferred. To the extent that a mutant will express from a different codon the same amino acids which are expressed by the above sequence, then such alternative sequence could be permitted.

The dipeptides which are encoded for by the sequence in the parenthesis will be an acidic amino acid, aspartic or glutamic, preferably glutamic, followed by a neutral amino acid, alanine and proline, particularly alanine.

In providing for useful DNA sequences which can be used for cassettes for expression, the following sequence can be conveniently employed:

25 $Tr-L-((R-S)_{r''}-(GAXYCX)_n-W-(Gene)_d)_y$

wherein:

Tr intends a DNA sequence encoding for the transcriptional regulatory signals; particularly the promoter and such other regulatory signals as operators, activators, cap signal, signals enhancing ribosomal binding, or other sequence involved with transcriptional or translational control. The Tr sequence will generally be at least about 100bp and not more than about 2000bp. Particularly useful is employing the Tr sequence associated with the leader sequence L, so that a DNA fragment can be employed which includes the transcriptional and translational signal sequences associated with the leader sequence endogenous to the host. Alternatively, one may employ other transcriptional and translational signals to provide for enhanced production of the expression product;

d is 0 or 1, being 1 when y is greater than 1;

n' is a whole number, generally ranging from 0 to 3, more usually being 0 or 2 to 3;

r'' is 1 or 2;

W intends a terminal deoxyribosyl-3' group, or a DNA sequence which by itself or, when n' is other than 0, in combination with the nucleotides to which it is joined, W defines a restriction site, having either a cohesive end or butt end, wherein W may have from 0 to about 20 nucleotides in the longest chain;

the remaining symbols having been defined previously.

Of particular interest is the following construct:

45

$(Tr)_a-L-(R-S)_{r''}-(GAXYCX)_n-GA\boxed{\overline{A}\overline{G}\overline{C}\overline{T}}$

wherein:

all of the symbols previously defined have the same definition;

a is 0 or 1 intending that the construct may or may not have the transcriptional and translational signals; the nucleotides indicated in the broken box are intended not to be present but to be capable of addition by adding an HindIII cleaved terminus to provide for the recreation of the sequence encoding for a dipeptide; and

n'' will be 0 to 2, where at least one of the Xs and Ys defines a nucleotide, so that the sequence in the parenthesis is other than the sequence GAAGCT.

The coding sequence of Gene* may be joined to the terminal T, providing that the coding sequence is in frame with the initiation codon and upon processing the first amino acid will be the correct amino acid for the mature polypeptide.

The 3'-terminus of Gene* can be manipulated much more easily and, therefore, it is desirable to provide a construct which allows for insertion of Gene* into a unique restriction site in the construct. Such a construct would provide for a restriction site with insertion of the Gene* into the restriction site to be in frame with the initiation codon. Such a construction can be symbolized as follows:

(Tr)_a-L-(R-S)_n-(GAXYCX)_m-W-(SC)_b-Te

wherein:

those symbols previously defined have the same definition;

SC are stop codons;

Te is a termination sequence balanced with the promoter Tr, and may include other signals, e.g. polyadenylation; and

b is an integer which will generally vary from about 0 to 4, more usually from 0 to 3, it being understood, that Gene* may include its own stop codons.

Illustrative of a sequence having the above formula is where W is the sequence GA and n is 2.

Of particular interest is where the sequence encoding the terminal dipeptide is taken together with W to define a linker or connector, which allows for recreation of the terminal sequence defining the dipeptide of the processing signal and encodes for the initial amino acids of Gene*, so that the codons are in frame with the initiation codon of the leader. The linker provides for a staggered or butt ended termination, desirably defining a restriction site in conjunction with the successive sequences of the Gene*. Upon ligation of the linker with Gene*, the codons of Gene* will be in frame with the initiation codon of the leader. In this manner, one can employ a synthetic sequence which may be joined to a restriction site in the processing signal sequence to recreate the processing signal, while providing the initial bases of the Gene* encoding for the N-terminal amino acids. By employing a synthetic sequence, the synthetic linker can be a tailored connector having a convenient restriction site near the 3'-terminus and the synthetic connector will then provide for the necessary codons for the 5'-terminus of the gene.

Alternatively, one could introduce a restriction endonuclease recognition site downstream from the processing signal to allow for cleavage and removal of superfluous bases to provide for ligation of the Gene* to the processing signal in frame with the initiation codon. Thus the first codon would encode for the N-terminal amino acid of the polypeptide. Where T is the first base of Gene*, one could introduce a restriction site where the recognition sequence is downstream from the cleavage site. For example, a Sau3A recognition sequence could be introduced immediately after the processing signal, which would allow for cleavage and linking of the Gene* with its initial codon in frame with the leader initiation codon. With restriction endonucleases which have the recognition sequence distal and downstream from the cleavage site e.g. HgaI, W could define such sequence which could include a portion of the processing signal sequences. Other constructions can also be employed, employing such techniques as primer repair and in vitro mutagenesis to provide for the convenient insertion of Gene* into the construct by introducing an appropriate restriction site.

The construct provides a portable sequence for insertion into vectors, which provide the desired replication system. As already indicated, in some instances, it may be desirable to replace the wild type promoter associated with the leader sequence with a different promoter. In yeast, promoters involved with enzymes in the glycolytic pathway can provide for high rates of transcription. These promoters are associated with such enzymes as phosphoglucose isomerase, phosphofructokinase, phosphotriose isomerase, phosphoglucose mutase, enolase, pyruvic kinase, glyceraldehyde-3-phosphate dehydrogenase, and alcohol dehydrogenase. These promoters may be inserted upstream from the leader sequence. The 5'-flanking region to the leader sequence may be retained or replaced with the 3'-sequence of the alternative promoter. Vectors can be prepared and have been reported which include promoters having convenient restriction sites downstream from the promoter for insertion of such constructs as described above.

The final construct will be an episomal element capable of stable maintenance in a host, particularly a fungal host such as yeast. The construct will include one or more replication systems, desirably two replication systems, allowing for maintenance in the expression host and cloning in a prokaryote. In addition, one or more markers for selection will be included, which will allow for selective pressure for maintenance of the episomal element in the host. Furthermore, the episomal element may be a high or low copy number, the copy number generally ranging from about 1 to 200. With high copy number episomal elements, there will generally be at least 10, preferably at least 20, and usually not exceeding about 150, more usually not exceeding about 100 copy number. Depending upon the Gene*, either high or low copy numbers may be desirable, depending upon the effect of the episomal element on the host. Where the presence of the expression product of the episomal element may have a deleterious effect on the viability

of the host, a low copy number may be indicated.

Various hosts may be employed, particularly mutants having desired properties. It should be appreciated that depending upon the rate of production of the expression product of the construct, the processing enzyme may or may not be adequate for processing at that level of production. Therefore, a mutant having enhanced production of the processing enzyme may be indicated or enhanced production of the enzyme may be provided by means of an episomal element. Generally, the production of the enzyme should be of a lower order than the production of the desired expression product.

Where one is using α -factor for secretion and processing, it would be appropriate to provide for enhanced production of the processing enzyme Dipeptidyl Amino Peptidase A, which appears to be the expression product of STE13. This enzyme appears to be specific for X-Ala- and X-Pro-sequences, where X in this instance intends an amino acid, particularly, the dicarboxylic acid amino acids.

Alternatively, there may be situations where intracellular processing is not desired. In this situation, it would be useful to have a ste13 mutant, where secretion occurs, but the product is not processed. In this manner, the product may be subsequently processed in vitro.

Host mutants which provide for controlled regulation of expression may be employed to advantage. For example, with the constructions of the subject invention where a fused protein is expressed, the transformants have slow growth which appears to be a result of toxicity of the fused protein. Thus, by inhibiting expression during growth, the host may be grown to high density before changing the conditions to permissive conditions for expression.

A temperature-sensitive sir mutant may be employed to achieve regulated expression. Mutation in any of the SIR genes results in a non-mating phenotype due to in situ expression of the normally silent MAT α and MAT α sequences present at the HML and HMR loci.

Furthermore, as already indicated, the Gene* may have a plurality of sequences in tandem, either the same or different sequences, with intervening processing signals. In this manner, the product may be processed in whole or in part, with the result that one will obtain the various sequences either by themselves or in tandem for subsequent processing. In many situations, it may be desirable to provide for different sequences, where each of the sequences is a subunit of a particular protein product.

The Gene* may encode for any type of polypeptide of interest. The polypeptide may be as small as an oligopeptide of 8 amino acids or may be 100,000 daltons or higher. Usually, single chains will be less than about 300,000 daltons, more usually less than about 150,000 daltons. Of particular interest are polypeptides of from about 5,000 to 150,000 daltons, more particularly of about 5,000 to 100,000 daltons. Illustrative polypeptides of interest include hormones and factors, such as growth hormone, somatomedins epidermal growth factor, the endocrine secretions, such as luteinizing hormone, thyroid stimulating hormone, oxytocin, insulin, vasopressin, renin, calcitonin, follicle stimulating hormone, prolactin, etc.; hematopoietic factors, e.g. erythropoietin, colony stimulating factor, etc.; lymphokines; globins, e.g. immunoglobulins; albumins; interferons, such as α , β and γ ; repressors; enzymes; endorphins e.g. β -endorphin, enkephalin, dynorphin, etc.

Having prepared the episomal elements containing the constructs of this invention, one may then introduce such element into an appropriate host. The manner of introduction is conventional, there being a wide variety of ways to introduce DNA into a host. Conveniently, spheroplasts are prepared employing the procedure of, for example, Hinnen et al., PNAS USA (1978) 75:1919-1933 or Stinchcomb et al., EP 0 045 573 A2. The transformants may then be grown in an appropriate nutrient medium and where appropriate, maintaining selective pressure on the transformants. Where expression is inducible, one can allow for growth of the yeast to high density and then induce expression. In those situations, where a substantial proportion of the product may be retained in the periplasmic space, one can release the product by treating the yeast cells with an enzyme such as zymolase or lyticase.

The product may be harvested by any convenient means, purifying the protein by chromatography, electrophoresis, dialysis, solvent-solvent extraction, etc.

In accordance with the subject invention, one can provide for secretion of a wide variety of polypeptides, so as to greatly enhance product yield, simplify purification, minimize degradation of the desired product, and simplify processing, equipment, and engineering requirements. Furthermore, utilization of nutrients based on productivity can be greatly enhanced, so that more economical and more efficient production of polypeptides may be achieved. Also, the use of yeast has many advantages in avoiding enterotoxins, which may be present with prokaryotes, and employing known techniques, which have been developed for yeast over long periods of time, which techniques include isolation of yeast products.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EP 0 116 201 B1

A synthetic sequence for human epidermal growth factor (EGF) based on the amino acid sequence of EGF reported by H. Gregory and B.M. Preston Int. J. Peptide Protein Res. 9, 107-118 (1977) was prepared, which had the following sequence.

5' AACTCCGACTCCGAATGTCCATTGTCCCACGACGGTTACTGTTTGACGACGGTGTTTGT
 3' TTGAGGCTGAGGCTTACAGGTAACAGGGTGCTGCCAATGACAAACGTGCTGCCACAAACA

ATGTACATCGAAGCTTTGGACAAGTACGCTTGTAACTGTGTTGTTGGTTACATCGGTGAA
 TACATGTAGCTTCGAAACCTGTTTCATGCGAACATTGACACAACAACCAATGTAGCCACTT

AGATGTCAATACAGAGACTTGAAGTGGTGGGAATTGAGATGA
 TCTACAGTTATGTCTCTGAACCTCACCACCCTTAACCTACT,

where 5' indicates the promoter proximal end of the sequence. The sequence was inserted into the EcoRI site of pBR328 to produce a plasmid p328EGF-1 and cloned.

Approximately 30µg of p328EGF-1 was digested with EcoRI and approximately 1µg of the expected 190 base pair EcoRI fragment was isolated. This was followed by digestion with the restriction enzyme HgaI. Two synthetic oligonucleotide connectors HindIII-HgaI and HgaI-SalI were then ligated to the 159 base pair HgaI fragment. The HgaI-HindIII linker had the following sequence:

AGCTGAAGCT
 CTTGATTGAG

This linker restores the α -factor processing signals interrupted by the HindIII digestion and joins the HgaI end at the 5'-end of the EGF gene to the HindIII end of pAB112.

The HgaI-SalI linker had the following sequence:

TGAGATGATAAG
 ACTATTCAGCT

This linker has two stop codons and joins the HgaI end at the 3'-end of the EGF gene to the SalI end of pAB112.

The resulting 181 base pair fragment was purified by preparative gel electrophoresis and ligated to 100ng of pAB112 which had been previously completely digested with the enzymes HindIII and SalI. Surprisingly, a deletion occurred where the codon for the 3rd and 4th amino acids of EGF, asp and ser, were deleted, with the remainder of the EGF being retained.

pAB112 is a plasmid containing a 1.75kb EcoRI fragment with the yeast α -factor gene cloned in the EcoRI site of pBR322 in which the HindIII and SalI sites had been deleted (pAB11). pAB112 was derived from plasmid pAB101 which contains the yeast α -factor gene as a partial Sau3A fragment cloned in the BamHI site of plasmid YEp24. pAB101 was obtained by screening a yeast genomic library in YEp24 using a synthetic 20-mer oligonucleotide probe (3'-GGCCGGTTGGTTACATGATT-5') homologous to the published α -factor coding region (Kurjan and Herskowitz, Abstracts 1981 Cold Spring Harbor meeting on the Molecular Biology of Yeasts, page 242).

The resulting mixture was used to transform E. coli HB101 cells and plasmid pAB201 obtained. Plasmid pAB201 (5µg) was digested to completion with the enzyme EcoRI and the resulting fragments were: a) filled in with DNA polymerase I Klenow fragment; b) ligated to an excess of BamHI linkers; and c) digested with BamHI. The 1.75kbp EcoRI fragment was isolated by preparative gel electrophoresis and approximately 100ng of the fragment was ligated to 100ng of pC1/1, which had been previously digested to completion with the restriction enzyme BamHI and treated with alkaline phosphatase.

Plasmid pC1/1 is a derivative of pJDB219, Beggs, Nature (1978) 275:104, in which the region corresponding to bacterial plasmid pMB9 in pJDB219 has been replaced by pBR322 in pC1/1. This mixture was used to transform E. coli HB101 cells. Transformants were selected by ampicillin resistance and their plasmids analyzed by restriction endonucleases. DNA from one selected clone (pYEGF-8) was prepared

and used to transform yeast AB103 cells. Transformants were selected by their *leu*⁺ phenotype.

Fifty milliliter cultures of yeast strain AB103 (α , pep 4-3, *leu* 2-3, *leu* 2-112, *ura* 3-52, *his* 4-580) transformed with plasmid pYEGF-8 (deposited at the American Type Culture Collection on 5th January 1983 and given ATCC Accession no. 20658) were grown at 30° in *-leu* medium to saturation (optical density at 600nm of 5) and left shaking at 30° for an additional 12 hr period. Cell supernatants were collected by centrifugation and analyzed for the presence of human EGF using the fibroblast receptor competition binding assay. The assay of EGF is based on the ability of both mouse and human EGF to compete with ¹²⁵I-labeled mouse EGF for binding sites on human foreskin fibroblasts. Standard curves can be obtained by measuring the effects of increasing quantities of EGF on the binding of a standard amount of ¹²⁵I-labeled mouse EGF. Under these conditions 2 to 20 ng of EGF are readily measurable. Details on the binding of ¹²⁵I-labeled epidermal growth factor to human fibroblasts have been described by Carpenter et al., J. Biol. Chem. 250, 4297 (1975). Using this assay it is found that the culture medium contains 7±1mg of human EGF per liter.

For further characterization, human EGF present in the supernatant was purified by absorption to the ion-exchange resin Biorex-70 and elution with HCl 10mM in 80% ethanol. After evaporation of the HCl and ethanol the EGF was solubilized in water. This material migrates as a single major protein of MW approx. 6,000 in 17.5% SDS gels, roughly the same as authentic mouse EGF (MW=6,000). This indicates that the α -factor leader sequence has been properly excised during the secretion process. Analysis by high resolution liquid chromatography (microbondapak C18, Waters column) indicates that the product migrates with a retention time similar to an authentic mouse EGF standard. However, protein sequencing by Edman degradation showed that the N-terminus retained the glu-ala sequence.

A number of other constructions were prepared using different constructions for joining hEGF to the α -factor secretory leader sequence, providing for different processing signals and site mutagenesis. In Fig. 2 a. through e. show the sequence of the fusions at the N-terminal region of hEGF, which sequence differ among several constructions. f. shows the sequences at the C-terminal region of hEGF, which is the same for all constructions. Synthetic oligonucleotide linkers used in these constructions are boxed.

These fusions were made as follows. Construction (a) was made as described above. Construction (b) was made in a similar way except that linker 2 was used instead of linker 1. Linker 2 modifies the α -factor processing signal by inserting an additional processing site (ser-leu-asn-lys-arg) immediately preceding the hEGF gene. The resulting yeast plasmid is named pY α EGF-22. Construction (c), in which the dipeptidyl aminopeptidase maturation site (glu-ala) has been removed, was obtained by *in vitro* mutagenesis of construction (a). A PstI-Sall fragment containing the α -factor leader-hEGF fusion was cloned in phage M13 and isolated in a single-stranded form. A synthetic 31-mer of sequence 5'-TCTTTGGATAAAAGAACTCCGACTCCCG-3' was synthesized and 70 picomoles were used as a primer for the synthesis of the second strand from 1 picomole of the above template by the Klenow fragment of DNA polymerase. After fill-in and ligation at 14° for 18 hrs, the mixture was treated with S₁ nuclease (5 units for 15 min) and used to transfect E. coli JM101 cells. Bacteriophage containing DNA sequences in which the region coding for (glu-ala) was removed were located by filter plaque hybridization using the ³²P-labeled primer as probe. RF DNA from positive plaques was isolated, digested with PstI and Sall and the resulting fragment inserted in pAB114 which had been previously digested to completion with Sall and partially with PstI and treated with alkaline phosphatase.

The plasmid pAB114 was derived as follows: plasmid pAB112 was digested to completion with HindIII and then religated at low (4μg/ml) DNA concentration and plasmid pAB113 was obtained in which three 63bp HindIII fragments have been deleted from the α -factor structural gene, leaving only a single copy of mature α -factor coding region. A BamHI site was added to plasmid pAB11 by cleavage with EcoRI, filling in of the overhanging ends by the Klenow fragment of DNA polymerase, ligation of BamHI linkers, cleavage with BamHI and religation to obtain pAB12. Plasmid pAB113 was digested with EcoRI, the overhanging ends filled in, and ligated to BamHI linkers. After digestion with BamHI the 1500bp fragment was gel-purified and ligated to pAB12 which had been digested with BamHI and treated with alkaline phosphatase. Plasmid pAB114, which contains a 1500bp BamHI fragment carrying the α -factor gene, was obtained. The resulting plasmid (pAB114 containing the above described construct) is then digested with BamHI and ligated into plasmid pC1/1.

The resulting yeast plasmid is named pY α EGF-23 and was deposited at the American Type Culture Collection on 12th August 1983 under ATCC Accession no. 40079. Construction (d), in which a new KpnI site was generated, was made as described for construction (c) except that the 36-mer oligonucleotide primer of sequence 5'-GGGTACCTTTGGATAAAAGAACTCCGACTCCGAAT-3' was used. The resulting yeast plasmid is named pY α EGF-24. Construction (e) was derived by digestion of the plasmid containing construction (d) with KpnI and Sall instead of linker 1 and 2. The resulting yeast plasmid is named pY α EGF-

25.

Yeast cells transformed with pY α EGF-22 were grown in 15 ml cultures. At the indicated densities or times, cultures were centrifuged and the supernatants saved and kept on ice. The cell pellets were washed in lysis buffer (0.1 Triton X-100, 10mM NaHPO₄ pH 7.5) and broken by vortexing (5min in 1min intervals with cooling on ice in between) in one volume of lysis buffer and one volume of glass beads. After centrifugation, the supernatants were collected and kept on ice. The amount of hEGF in the culture medium and cell extracts was measured using the fibroblast receptor binding competition assay. Standard curves were obtained by measuring the effects of increasing quantities of mouse EGF on the binding of a standard amount ¹²⁵I-labeled mouse EGF.

Proteins were concentrated from the culture media by absorption on Bio-Rex 70 resin and elution with 0.01 HCl in 80% ethanol and purified by high performance liquid chromatography (HPLC) on a reverse phase C18 column. The column was eluted at a flow rate of 4ml/min with a linear gradient of 5% to 80% acetonitrile containing 0.2% trifluoroacetic acid in 60min. Proteins (200-800 picomoles) were sequenced at the amino-terminal end by the Edman degradation method using a gas-phase protein sequencer Applied Biosystems model 470A. The normal PROTFA program was used for all the analyses. Dithiothreitol was added to S2 (ethyl acetate: 20mg/liter) and S3 (butyl chloride: 10mg/liter) immediately before use. All samples were treated with 1N HCl in methanol at 40° for 15min to convert PTH-aspartic acid and PTH-glutamic acid to their methyl esters. All PTH-amino acid identifications were performed by reference to retention times on a IBM CN HPLC column using a known mixture of PTH-amino acids as standards.

Secretion from pY α EGF-22 gave a 4:1 mole ratio of native N-terminus hEGF to glu-ala terminated hEGF, while secretion from pY α EGF-23-25 gave only native N-terminated hEGF. Yields of hEGF ranged from 5 to 8 μ g/ml measured either as protein or in a receptor binding assay.

The strain JRY188 (MAT sir3-8 leu2-3 leu2-112 trp1 ura3 his4 rme) was transformed with pY α EGF-21 and leucine prototrophs selected at 37°. Saturated cultures were then diluted 1/100 in fresh medium and grown in leucine selective medium at permissive (24°) and non-permissive (36°) temperatures and culture supernatants were assayed for the presence of hEGF as described above. The results are shown in the following table.

Regulated synthesis and secretion of hEGF in transformed yeast sir3 temperature-sensitive mutants.

Temperature	Transformant	O.D. 650	hEGF(μ g/ml)
36°	3a	3.5	0.010
		5.4	0.026
	3b	3.6	0.020
		6.4	0.024
24°	3a	0.4	34
		1.3	145
		2.1	1075
		4.0	3250
	3b	0.4	32
		1.4	210
		2.2	1935
		4.2	4600

These results indicate that the hybrid α -factor/EGF gene is being expressed under mating type regulation, even though it is present on a high copy number plasmid.

In accordance with the subject invention, novel constructs are provided which may be inserted into

vectors to provide for expression of polypeptides having an N-terminal leader sequence and one or more processing signals to provide for secretion of the polypeptide as well as processing to result in a mature polypeptide product free of superfluous amino acids. Thus, one can obtain a polypeptide having the identical sequence to a naturally occurring polypeptide. In addition, because the polypeptide can be produced in yeast, glycosylation can occur, so that products can be obtained which are identical to the naturally occurring products. Furthermore, because the product is secreted, greatly enhanced yields can be obtained based on cell population and processing and purification are greatly simplified. In addition, employing mutant hosts, expression can be regulated to be turned off or on, as desired.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

Claims for the following Contracting States : BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE

1. A DNA construct encoding a protein foreign to yeast, the amino acid sequence of said protein comprising at least a yeast alpha-factor leader sequence fragment that provides for secretion linked to a heterologous polypeptide sequence, said protein also containing yeast processing signals between said alpha-factor leader sequence fragment and said heterologous polypeptide for processing said protein into said heterologous polypeptide.
2. A DNA construct according to Claim 1 further comprising a yeast promoter at the 5' end.
3. A DNA construct according to any of Claims 1 - 2 wherein said heterologous polypeptide is a mammalian protein.
4. A DNA construct comprising a sequence comprising the formula:
5'-Tr-L-Sp-Gene*-Te-3'
wherein:
Tr is a yeast promoter sequence;
L encodes at least a yeast alpha-factor leader sequence fragment that provides for secretion;
Sp is a spacer sequence encoding processing signals for processing the precursor polypeptide encoded by L-Sp-Gene* into the polypeptide encoded by Gene*;
Gene* encodes a polypeptide foreign to yeast; and Te is a transcription termination sequence balanced with Tr.
5. The DNA construct of Claim 4 wherein Tr comprises a yeast alpha-factor promoter sequence.
6. The DNA construct according to either of Claims 4 - 5 wherein Sp contains the sequence 5'-R₁-R₂-3' immediately adjacent to the sequence Gene*, R₁ being a codon for lysine or arginine, R₂ being a codon for arginine but does not encode a processing signal for dipeptidylaminopeptidase A.
7. A DNA construct according to Claim 6 wherein Sp is 5'-R₁-R₂-3'.
8. A DNA construct according to Claim 4 wherein Gene* encodes a mammalian protein.
9. A DNA construct according to Claim 5 wherein Gene* encodes a mammalian protein.
10. A DNA construct according to Claim 6 wherein Gene* encodes a mammalian protein.
11. A DNA construct according to Claim 8 wherein said mammalian protein is human epidermal growth factor.
12. A DNA construct according to Claim 9 wherein said mammalian protein is human epidermal growth factor.

13. A DNA construct according to Claim 10 wherein said mammalian protein is human epidermal growth factor.
14. An episomal expression element comprising a DNA construct according to Claim 8 and a replication system providing stable maintenance in a yeast host.
15. An episomal expression element comprising a DNA construct according to Claim 9 and a replication system providing stable maintenance in a yeast host.
16. An episomal expression element comprising a DNA construct according to Claim 10 and a replication system providing stable maintenance in a yeast host.
17. A method for producing a recombinant polypeptide in yeast and having said polypeptide secreted into the culture medium, said method comprising:
 - providing a yeast host transformed by a DNA construct according to Claim 4;
 - growing in said culture medium said transformed yeast under conditions whereby the precursor polypeptide encoded by 5'-L-Sp-Gene'-3' is expressed, at least partially processed into a polypeptide having the sequence encoded by Gene', and secreted into said culture medium;
 - and recovering from said culture medium said secreted polypeptide.
18. A method according to Claim 17 wherein Tr comprises a yeast alpha-factor promoter sequence.
19. A method according to either Claim 17 or 18 wherein Sp contains the sequence 5'-R₁-R₂-3' immediately adjacent to the sequence Gene', R₁ being a codon for lysine or arginine, R₂ being a codon for arginine, but does not encode a processing signal for dipeptidylaminopeptidase A.
20. A method according to Claim 19 wherein S is 5'-R₁-R₂-3'.
21. A method according to any of Claims 17 - 20 wherein Gene' encodes a mammalian polypeptide.
22. A method according to Claim 21 wherein said mammalian polypeptide is epidermal growth factor.
23. A method according to any of Claims 17 - 20 wherein said yeast is strain AB 103, ATCC No. 20 858.
24. Plasmid pYEGF8, ATCC Accession No. 20858.
25. Plasmid pYαEGF 23., ATCC Accession No. 40079
26. A method for producing a recombinant polypeptide comprising:
 - providing a yeast host transformed by a DNA construct encoding a protein foreign to yeast, the amino acid sequence of said protein comprising at least a yeast alpha-factor leader sequence fragment that provides for secretion linked to a heterologous polypeptide sequence, said protein also containing yeast processing signals between said alpha-factor leader sequence fragment and said heterologous polypeptide for processing said protein into said heterologous polypeptide;
 - growing in said culture medium said transformed yeast host under conditions whereby said protein foreign to yeast is expressed, at least partially processed into said heterologous polypeptide, and secreted into said culture medium;
 - and recovering from said culture medium said secreted heterologous polypeptide.
27. A method according to Claim 26 wherein said heterologous polypeptide is a mammalian protein.
28. A method according to Claim 26 wherein said yeast alpha-factor is Saccharomyces alpha-factor.
29. A method according to claim 26 wherein said heterologous polypeptide is processed intracellularly or extracellularly to provide a mature polypeptide.
30. A method according to claim 29 wherein said mature polypeptide is selected from the group consisting of growth hormone, somatomedins, epidermal growth factor, insulin, renin, calcitonin, albumins and

interferons.

31. A method according to any of claims 17, 19 or 21 wherein the polypeptide encoded by Gene* is processed intracellularly or extracellularly to provide a mature polypeptide.

32. A method according to claim 31 wherein said mature polypeptide is selected from the group consisting of growth hormone, somatomedins, epidermal growth factor, insulin, renin, calcitonin, albumins and interferons.

33. A method according to any of claims 26 to 32 wherein said yeast is strain AB 103, ATCC. No. 20658.

34. A host cell transformed by a DNA construct according to any of claims 1, 4, 6 or 7.

Claims for the following Contracting State : AT

1. A method for producing a recombinant polypeptide

comprising:

providing a yeast host transformed by a DNA construct encoding a protein foreign to yeast, the amino acid sequence of said protein comprising at least a yeast alpha-factor leader sequence fragment that provides for secretion linked to a heterologous polypeptide sequence, said protein also containing yeast processing signals between said alpha-factor leader sequence fragment and said heterologous polypeptide for processing said protein into said heterologous polypeptide;

growing in said culture medium said transformed yeast host under conditions whereby said protein foreign to yeast is expressed, at least partially processed into said heterologous polypeptide, and secreted into said culture medium;

and recovering from said culture medium said secreted heterologous polypeptide.

2. A method according to Claim 1 wherein said heterologous polypeptide is a mammalian protein.

3. A method according to any of Claims 1 - 2 wherein said DNA construct comprises a sequence comprising the formula:

5'-Tr-L-Sp-Gene*-Te-3'

wherein:

- Tr is a yeast promoter sequence;
- L encodes said yeast alpha-factor leader sequence fragment;
- Sp is a spacer sequence encoding said yeast processing signals for processing the precursor polypeptide encoded by L-Sp-Gene* into the polypeptide encoded by Gene*;
- Gene* encodes said heterologous polypeptide; and
- Te is a transcription termination sequence balanced with Tr.

4. A method according to Claim 3 wherein Sp contains the sequence 5'-R₁-R₂-3' immediately adjacent to the sequence Gene*, R₁ being a codon for lysine or arginine, R₂ being a codon for arginine but does not encode a processing signal for dipeptidylaminopeptidase A.

5. A method according to Claim 4 wherein Sp is 5'-R₁-R₂-3'.

6. A method according to Claim 3 wherein Tr comprises a yeast alpha-factor promoter sequence.

7. A method according to Claim 1 wherein said yeast alpha-factor is *Saccharomyces* alpha-factor.

8. A method according to Claim 1 wherein said yeast alpha-factor is *S. cerevisiae* alpha-factor.

9. A method according to Claim 2 wherein said mammalian protein is human epidermal growth factor.

10. A method according to Claim 4 wherein Gene* encodes human epidermal growth factor.

11. A method according to Claim 5 wherein Gene^{*} encodes human epidermal growth factor.
12. A method according to Claim 7 wherein said heterologous polypeptide comprises human epidermal growth factor.
- 5 13. A method according to Claim 8 wherein said heterologous polypeptide comprises human epidermal growth factor.
- 10 14. A method according to any of claims 1, 7, or 8 wherein said heterologous polypeptide is processed intracellularly or extracellularly to provide a mature polypeptide.
- 15 15. A method according to claim 4 wherein said heterologous polypeptide is processed intracellularly or extracellularly to provide a mature polypeptide.
- 16 16. A method according to claim 15 wherein said mature polypeptide is selected from the group consisting of growth hormone, somatomedins, epidermal growth factor, insulin, renin, calcitonin, albumins and interferons.
- 20 17. A method according to claim 16 wherein said mature polypeptide is selected from the group consisting of growth hormone, somatomedins, epidermal growth factor, insulin, renin, calcitonin, albumins and interferons.
- 25 18. A method according to any of Claims 1, 7, 8 and 15 to 17 wherein said yeast is strain AB 103, ATCC No. 20 658.

Revendications

Revendications pour les Etats contractant suivants : BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE

- 30 1. Construction d'ADN codant pour une protéine étrangère à une levure, la séquence d'acides aminés de cette protéine comprenant au moins un fragment de séquence de tête de facteur alpha de levure qui pourvoit à la sécrétion lié à une séquence de polypeptide hétérologue, cette protéine contenant aussi des signaux de maturation moléculaire de levure entre ce fragment de séquence de tête de facteur alpha et ce polypeptide hétérologue pour la maturation moléculaire de cette protéine dans ce polypeptide hétérologue.
- 35 2. Construction d'ADN suivant la revendication 1, comprenant de plus un promoteur de levure à l'extrémité 5'.
- 40 3. Construction d'ADN suivant la revendication 1 ou la revendication 2, dans laquelle ce polypeptide hétérologue est une protéine de mammifère.
4. Construction d'ADN comprenant une séquence comprenant la formule :
 - 45 5'-Tr-L-Sp-Gène^{*}-Te-3'
 - dans laquelle :
 - Tr est une séquence promoteur de levure;
 - L code au moins pour un fragment de séquence de tête de facteur alpha de levure qui pourvoit à la sécrétion;
 - 50 Sp est une séquence espaceur codant pour des signaux de maturation moléculaire pour la maturation moléculaire du polypeptide précurseur codé par L-Sp-Gène^{*} en polypeptide codé par Gène^{*};
 - Gène^{*} code pour un polypeptide étranger à une levure; et
 - 55 Te est une séquence de terminaison équilibrée par Tr.
5. Construction d'ADN suivant la revendication 4, dans laquelle Tr comprend une séquence promoteur de facteur alpha de levure.

6. Construction d'ADN suivant la revendication 4 ou la revendication 5, dans laquelle Sp contient la séquence 5'-R₁-R₂-3' immédiatement adjacente à la séquence Gène*, R₁ étant un codon pour la lysine ou l'arginine, R₂ étant un codon pour l'arginine, mais qui ne code pas pour un signal de maturation moléculaire pour la dipeptidylaminopeptidase A.
- 5 7. Construction d'ADN suivant la revendication 6, dans laquelle Sp est 5'-R₁-R₂-3'.
8. Construction d'ADN suivant la revendication 4, dans laquelle Gène* code pour une protéine de mammifère.
- 10 9. Construction d'ADN suivant la revendication 5, dans laquelle Gène* code pour une protéine de mammifère.
10. Construction d'ADN suivant la revendication 6, dans laquelle Gène* code pour une protéine de mammifère.
- 15 11. Construction d'ADN suivant la revendication 8, dans laquelle cette protéine de mammifère est un facteur de croissance épidermique humain.
- 20 12. Construction d'ADN suivant la revendication 9, dans laquelle cette protéine de mammifère est un facteur de croissance épidermique humain.
13. Construction d'ADN suivant la revendication 10, dans laquelle cette protéine de mammifère est un facteur de croissance épidermique humain.
- 25 14. Élément d'expression épisomique comprenant une construction d'ADN suivant la revendication 8 et un système de répllication pourvoyant à un maintien stable dans une levure-hôte.
- 30 15. Élément d'expression épisomique comprenant une construction d'ADN suivant la revendication 9 et un système de répllication pourvoyant à un maintien stable dans une levure-hôte.
16. Élément d'expression épisomial comprenant une construction d'ADN suivant la revendication 10 et un système de répllication pourvoyant à un maintien stable dans une levure-hôte.
- 35 17. Méthode pour produire un polypeptide recombinant dans une levure et avoir une sécrétion de ce polypeptide dans le milieu de culture, cette méthode comprenant:
 - la fourniture d'une levure-hôte transformée par une construction d'ADN suivant la revendication 4;
 - la croissance dans ce milieu de culture de cette levure transformée dans des conditions dans lesquelles le polypeptide précurseur codé par 5'-L-Sp-Gène*-3' est exprimé, soumis au moins partiellement à une maturation moléculaire en un polypeptide ayant la séquence codée par Gène* et sécrété dans ce milieu de culture;
 - et la récupération à partir de ce milieu de culture de ce polypeptide sécrété.
- 40 18. Méthode suivant la revendication 17, dans laquelle Tr comprend une séquence promoteur de facteur alpha de levure.
- 45 19. Méthode suivant la revendication 17 ou la revendication 18, dans laquelle Sp contient la séquence 5'-R₁-R₂-3' immédiatement adjacente à la séquence Gène*, R₁ étant un codon pour la lysine ou l'arginine, R₂ étant un codon pour l'arginine, mais qui ne code pas pour un signal de maturation moléculaire pour la dipeptidylaminopeptidase A.
- 50 20. Méthode suivant la revendication 19, dans laquelle Sp est 5'-R₁-R₂-3'.
21. Méthode suivant l'une quelconque des revendications 17 à 20, dans laquelle Gène* code pour un polypeptide de mammifère.
- 55 22. Méthode suivant la revendication 21, dans laquelle ce polypeptide de mammifère est un facteur de croissance épidermique.

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23. Méthode suivant l'une quelconque des revendications 17 à 20, dans laquelle cette levure est la souche AB 103, ATCC No. 20 658.
24. Plasmide pYEGF8, No d'accès ATCC No. 20 658.
25. Plasmide pYaEGF23, No d'accès ATCC No. 40 079.
26. Méthode pour produire un polypeptide recombinant comprenant:
la fourniture d'une levure-hôte transformée par une construction d'ADN codant pour une protéine étrangère à la levure, la séquence d'acides aminés de cette protéine comprenant au moins un fragment de séquence de tête de facteur alpha de levure qui pourvoit à la sécrétion lié à une séquence de polypeptide hétérologue, cette protéine contenant aussi des signaux de maturation moléculaire de levure entre ce fragment de séquence de tête de facteur alpha et ce polypeptide hétérologue pour la maturation moléculaire de cette protéine en ce polypeptide hétérologue;
la croissance dans ce milieu de culture de cette levure-hôte transformée dans des conditions dans lesquelles cette protéine étrangère à la levure est exprimée, soumise au moins partiellement à une maturation moléculaire en ce polypeptide hétérologue et sécrétée dans ce milieu de culture;
et la récupération à partir de ce milieu de culture de ce polypeptide hétérologue sécrété.
27. Méthode suivant la revendication 26, dans laquelle ce polypeptide hétérologue est une protéine de mammifère.
28. Méthode suivant la revendication 26, dans laquelle ce facteur alpha de levure est le facteur alpha de *Saccharomyces*.
29. Méthode suivant la revendication 26, dans laquelle ce polypeptide hétérologue est soumis à une maturation moléculaire de façon intracellulaire ou extracellulaire pour fournir un polypeptide mature.
30. Méthode suivant la revendication 29, dans laquelle ce polypeptide mature est choisi dans le groupe consistant en hormone de croissance, somatomédines, facteur de croissance épidermique, insuline, rénine, calcitonine, albumines et interférons.
31. Méthode suivant l'une quelconque des revendications 17, 19 ou 21, dans laquelle le polypeptide codé par Gène* est soumis à une maturation moléculaire de façon intracellulaire ou extracellulaire pour fournir un polypeptide mature.
32. Méthode suivant la revendication 31, dans laquelle ce polypeptide mature est choisi dans le groupe consistant en hormone de croissance, somatomédines, facteur de croissance épidermique, insuline, rénine, calcitonine, albumines et interférons.
33. Méthode suivant l'une quelconque des revendications 26 à 32, dans laquelle cette levure est la souche AB 103, ATCC No. 20 658.
34. Cellule-hôte transformée par une construction d'ADN suivant l'une quelconque des revendications 1, 4, 6 ou 7.

Revendications pour l'Etat contractant suivant : AT

1. Méthode pour produire un polypeptide recombinant comprenant:
la fourniture d'une levure-hôte transformée par une construction d'ADN codant pour une protéine étrangère à la levure, la séquence d'acides aminés de cette protéine comprenant au moins un fragment de séquence de tête de facteur alpha de levure qui pourvoit à la sécrétion lié à une séquence de polypeptide hétérologue, cette protéine contenant aussi des signaux de maturation moléculaire de levure entre ce fragment de séquence de tête de facteur alpha et ce polypeptide hétérologue pour la maturation moléculaire de cette protéine en ce polypeptide hétérologue;
la croissance dans ce milieu de culture de cette levure-hôte transformée dans des conditions dans lesquelles cette protéine étrangère à la levure est exprimée, soumise au moins partiellement à une maturation moléculaire en ce polypeptide hétérologue et sécrétée dans ce milieu de culture;

et la récupération à partir de ce milieu de culture de ce polypeptide hétérologue sécrété.

2. Méthode suivant la revendication 1, dans laquelle ce polypeptide hétérologue est une protéine de mammifère.
- 5 3. Méthode suivant la revendication 1 ou la revendication 2, dans laquelle cette construction d'ADN comprend une séquence comprenant la formule :

5'-Tr-L-Sp-Gène*-Te-3'
10 dans laquelle :
Tr est une séquence promoteur de levure;
L code pour ce fragment de séquence de tête de facteur alpha de levure;
Sp est une séquence espaceur codant pour ces signaux de maturation moléculaire pour la maturation moléculaire du polypeptide précurseur codé par L-Sp-Gène* en polypeptide codé par Gène*;
15 Gène* code pour ce polypeptide hétérologue; et
Te est une séquence de terminaison équilibrée par Tr.
- 20 4. Méthode suivant la revendication 3, dans laquelle Sp contient la séquence 5'-R₁-R₂-3' immédiatement adjacente à la séquence Gène*, R₁ étant un codon pour la lysine ou l'arginine, R₂ étant un codon pour l'arginine, mais qui ne code pas pour un signal de maturation moléculaire pour la dipeptidylaminopeptidase A.
- 25 5. Méthode suivant la revendication 4, dans laquelle Sp est 5'-R₁-R₂-3'.
6. Méthode suivant la revendication 3, dans laquelle Tr comprend une séquence promoteur de facteur alpha de levure.
- 30 7. Méthode suivant la revendication 1, dans laquelle ce facteur alpha de levure est un facteur alpha de *Saccharomyces*.
8. Méthode suivant la revendication 1, dans laquelle ce facteur alpha de levure est un facteur alpha de *S. cerevisiae*.
- 35 9. Méthode suivant la revendication 2, dans laquelle cette protéine de mammifère est un facteur de croissance épidermique humain.
- 40 10. Méthode suivant la revendication 4, dans laquelle Gène* code pour un facteur de croissance épidermique humain.
11. Méthode suivant la revendication 5, dans laquelle Gène* code pour un facteur de croissance épidermique humain.
- 45 12. Méthode suivant la revendication 7, dans laquelle ce polypeptide hétérologue comprend un facteur de croissance épidermique humain.
13. Méthode suivant la revendication 8, dans laquelle ce polypeptide hétérologue comprend un facteur de croissance épidermique humain.
- 50 14. Méthode suivant l'une quelconque des revendications 1, 7 ou 8, dans laquelle ce polypeptide hétérologue est soumis à une maturation moléculaire de façon intracellulaire ou extracellulaire pour fournir un polypeptide mature.
- 55 15. Méthode suivant la revendication 4, dans laquelle ce polypeptide hétérologue est soumis à une maturation moléculaire de façon intracellulaire ou extracellulaire pour fournir un polypeptide mature.
16. Méthode suivant la revendication 15, dans laquelle ce polypeptide mature est choisi dans le groupe

consistant en hormone de croissance, somatomédines, facteur de croissance épidermique, insuline, rénine, calcitonine, albumines et interférons.

17. Méthode suivant la revendication 16, dans laquelle ce polypeptide mature est choisi dans le groupe consistant en hormone de croissance, somatomédines, facteur de croissance épidermique, insuline, rénine, calcitonine, albumines et interférons.
18. Méthode suivant l'une quelconque des revendications 1, 7, 8 et 15 à 17, dans laquelle cette levure est la souche AB 103, ATCC No. 20 658.

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Patentansprüche

Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE

1. DNA-Konstrukt, das ein zu Hefe fremdes Protein kodiert, wobei die Aminosäuresequenz des Proteins zumindest ein die Sekretion ermöglichendes Hefe- α -Faktor-Leader-Sequenz-Fragment, verknüpft mit einer heterologen Polypeptidsequenz, umfaßt und das Protein zum Processing des Proteins in das heterologe Polypeptid weiterhin Hefe-Processing-Signale zwischen dem α -Faktor-Leader-Sequenz-Fragment und dem heterologen Polypeptid enthält.
2. DNA-Konstrukt gemäß Anspruch 1, das weiterhin an dem 5'-Ende einen Hefe-Promotor umfaßt.
3. DNA-Konstrukt gemäß einem jeden der Ansprüche 1 bis 2, bei dem das heterologe Polypeptid ein Säugetierprotein ist.
4. DNA-Konstrukt, das eine Sequenz umfaßt, die die Formel:
$$5'\text{-Tr-L-Sp-Gene}^*\text{-Te-3'}$$
umfaßt, in der:
Tr eine Hefe-Promotor-Sequenz ist;
L zumindest ein die Sekretion ermöglichendes Hefe- α -Faktor-Leader-Sequenz-Fragment kodiert;
Sp eine Spacersequenz ist, die Processing-Signale zum Processing des durch L-Sp-Gene* kodierten Vorläufer-Polypeptids in das durch Gene* kodierte Polypeptid kodiert;
Gene* ein zu Hefe fremdes Polypeptid kodiert; und
Te eine mit Tr balancierte Terminations-Sequenz ist.
5. DNA-Konstrukt nach Anspruch 4, bei dem Tr eine Hefe- α -Faktor-Promotor-Sequenz umfaßt.
6. DNA-Konstrukt gemäß einem der Ansprüche 4 bis 5, bei dem Sp die Sequenz 5'-R₁-R₂-3' unmittelbar neben der Sequenz Gene* enthält, wobei R₁ ein Kodon für Lysin oder Arginin und R₂ ein Kodon für Arginin ist, aber nicht ein Processing-Signal für Dipeptidylaminopeptidase A kodiert.
7. DNA-Konstrukt gemäß Anspruch 6, bei dem Sp 5'-R₁-R₂-3' ist.
8. DNA-Konstrukt gemäß Anspruch 4, bei dem Gene* ein Säugetierprotein kodiert.
9. DNA-Konstrukt gemäß Anspruch 5, bei dem Gene* ein Säugetierprotein kodiert.
10. DNA-Konstrukt gemäß Anspruch 6, bei dem Gene* ein Säugetierprotein kodiert.
11. DNA-Konstrukt gemäß Anspruch 8, bei dem das Säugetierprotein ein humaner epidermaler Wachstumsfaktor ist.

12. DNA-Konstrukt gemäß Anspruch 9, bei dem das Säugetierprotein ein humaner epidermaler Wachstumsfaktor ist.
13. DNA-Konstrukt gemäß Anspruch 10, bei dem das Säugetierprotein ein humaner epidermaler Wachstumsfaktor ist.
14. Episomales Expressionselement, das ein DNA-Konstrukt gemäß Anspruch 8 und ein ein stabiles Erhaltenbleiben in einem Hefewirt ermöglichendes Replikationssystem umfaßt.
15. Episomales Expressionselement, das ein DNA-Konstrukt gemäß Anspruch 9 und ein ein stabiles Erhaltenbleiben in einem Hefewirt ermöglichendes Replikationssystem umfaßt.
16. Episomales Expressionselement, das ein DNA-Konstrukt gemäß Anspruch 10 und ein ein stabiles Erhaltenbleiben in einem Hefewirt ermöglichendes Replikationssystem umfaßt.
17. Verfahren zur Herstellung eines rekombinanten Polypeptids in Hefe und zum Erhalt der Sekretion des Polypeptids in das Kulturmedium, bei dem:

ein mit einem DNA-Konstrukt gemäß Anspruch 4 transformierter Hefewirt bereitgestellt wird;

die transformierte Hefe in dem Kulturmedium unter Bedingungen gezüchtet wird, bei denen das durch 5'-L-Sp-Gene*-3' kodierte Vorläufer-Polypeptid exprimiert, mindestens teilweise in ein die von Gene* kodierte Sequenz aufweisendes Polypeptid weiterverarbeitet und in das Kulturmedium sekretiert wird;

und das sekretierte Polypeptid aus dem Kulturmedium wiedergewonnen wird.
18. Verfahren gemäß Anspruch 17, bei dem Tr eine Hefe- α -Faktor-Promotor-Sequenz umfaßt.
19. Verfahren gemäß einem der Ansprüche 17 oder 18, bei dem Sp die Sequenz 5'-R₁-R₂-3' unmittelbar neben der Sequenz Gene* enthält, wobei R₁ ein Kodon für Lysin oder Arginin und R₂ ein Kodon für Arginin ist, aber nicht ein Processing-Signal für Dipeptidylaminopeptidase A kodiert.
20. Verfahren gemäß Anspruch 19, bei dem S 5'-R₁-R₂-3' ist.
21. Verfahren gemäß einem jeden der Ansprüche 17 bis 20, bei dem Gene* ein Säugetierpolypeptid kodiert.
22. Verfahren gemäß Anspruch 21, bei dem das Säugetierpolypeptid ein epidermaler Wachstumsfaktor ist.
23. Verfahren nach einem jeden der Ansprüche 17 bis 20, bei dem die Hefe der Stamm AB 103, ATCC-Nr. 20 658, ist.
24. Plasmid pYEGF8, ATCC-Zugangs-Nr. 20658.
25. Plasmid pY α EGF 23., ATCC-Zugangs-Nr. 40079.
26. Verfahren zur Herstellung eines rekombinanten Polypeptids, mit den Schritten:

Bereitstellen eines Hefewirts, der mit einem DNA-Konstrukt, das ein zu Hefe fremdes Protein kodiert, transformiert ist, wobei die Aminosäuresequenz des Proteins zumindest ein die Sekretion ermöglichendes Hefe- α -Faktor-Leader-Sequenz-Fragment verknüpft mit einer heterologen Polypeptidsequenz umfaßt, und das Protein zum Processing des Proteins in das heterologe Polypeptid weiterhin Hefe-Processing-Signale zwischen dem α -Faktor-Leader-Sequenz-Fragment und dem heterologen Polypeptid enthält;

Züchten des transformierten Hefewirts in dem Kulturmedium unter Bedingungen, bei denen das zu Hefe fremde Protein exprimiert, mindestens teilweise zu dem heterologen Polypeptid weiterverarbeitet und in das Kulturmedium sekretiert wird;

und Zurückgewinnen des heterologen Polypeptids aus dem Kulturmedium.

27. Verfahren gemäß Anspruch 26, bei dem das heterologe Polypeptid ein Säugetierprotein ist.
- 5 28. Verfahren gemäß Anspruch 26, bei dem der Hefe- α -Faktor der *Saccharomyces*- α -Faktor ist.
29. Verfahren gemäß Anspruch 26, bei dem das heterologe Polypeptid zum Erhalt eines reifen Polypeptids intrazellulär oder extrazellulär weiterverarbeitet wird.
- 10 30. Verfahren gemäß Anspruch 29, bei dem das reife Polypeptid aus der von dem Wachstumshormon, Somatomedinen, dem epidermalen Wachstumsfaktor, Insulin, Renin, Calcitonin, Albuminen und Interferonen gebildeten Gruppe ausgewählt ist.
- 15 31. Verfahren gemäß einem jeden der Ansprüche 17, 19 oder 21, bei dem das von Gene* kodierte Polypeptid zum Erhalt eines reifen Polypeptids intrazellulär oder extrazellulär weiterverarbeitet wird.
32. Verfahren gemäß Anspruch 31, bei dem das reife Polypeptid aus der von dem Wachstumshormon, Somatomedinen, dem epidermalen Wachstumsfaktor, Insulin, Renin, Calcitonin, Albuminen und Interferonen gebildeten Gruppe ausgewählt ist.
- 20 33. Verfahren gemäß einem jeden der Ansprüche 26 bis 32, bei dem die Hefe der Stamm AB 103, ATCC.-Nr. 20658, ist.
- 25 34. Wirtszelle, die mit einem DNA-Konstrukt gemäß einem jeden der Ansprüche 1, 4, 6 oder 7 transformiert ist.

Patentansprüche für folgenden Vertragsstaat : AT

1. Verfahren zur Herstellung eines rekombinanten Polypeptids, mit den Schritten:
 - 30 Bereitstellen eines Hefewirts, der mit einem DNA-Konstrukt, das ein zu Hefe fremdes Protein kodiert, transformiert ist, wobei die Aminosäuresequenz des Proteins zumindest ein die Sekretion ermöglichendes Hefe- α -Faktor-Leader-Sequenz-Fragment verknüpft mit einer heterologen Polypeptidsequenz umfaßt, und das Protein zum Processing des Proteins in das heterologe Polypeptid weiterhin Hefe-Processing-Signale zwischen dem α -Faktor-Leader-Sequenz-Fragment und dem heterologen Polypeptid enthält;
 - 35 Züchten des transformierten Hefewirts in dem Kulturmedium unter Bedingungen, bei denen das zu Hefe fremde Protein exprimiert, mindestens teilweise zu dem heterologen Polypeptid weiterverarbeitet und in das Kulturmedium sekretiert wird;
 - 40 und Zurückgewinnen des heterologen Polypeptids aus dem Kulturmedium.
2. Verfahren gemäß Anspruch 1, bei dem das heterologe Polypeptid ein Säugetierprotein ist.
- 45 3. Verfahren gemäß einem jeden der Ansprüche 1 bis 2, bei dem das DNA-Konstrukt eine Sequenz umfaßt, die die Formel:

5'-Tr-L-Sp-Gene*-Te-3'

- 50 umfaßt, in der:

Tr eine Hefe-Promotor-Sequenz ist;

L zumindest ein die Sekretion ermöglichendes Hefe- α -Faktor-Leader-Sequenz-Fragment kodiert;

Sp eine Spacersequenz ist, die die Processing-Signale zum Processing des durch L-Sp-Gene* kodierten Vorläufer-Polypeptids in das durch Gene* kodierte Polypeptid kodiert;

Gene* das heterologe Polypeptid kodiert; und

Te eine mit Tr balancierte Terminations-Sequenz ist.

- 5
4. Verfahren gemäß Anspruch 3, bei dem Sp die Sequenz 5'-R₁-R₂-3' unmittelbar neben der Sequenz Gene* enthält, R₁ ein Kodon für Lysin oder Arginin und R₂ ein Kodon für Arginin ist, aber nicht ein Processing-Signal für Dipeptidylaminopeptidase A kodiert.
- 10
5. Verfahren gemäß Anspruch 4, bei dem Sp 5'-R₁-R₂-3' ist.
6. Verfahren gemäß Anspruch 3, bei dem Tr eine Hefe- α -Faktor-Promotor-Sequenz einschließt.
7. Verfahren gemäß Anspruch 1, bei dem der Hefe- α -Faktor ein *Saccharomyces*- α -Faktor ist.
- 15
8. Verfahren gemäß Anspruch 1, bei dem der Hefe- α -Faktor der *S. cerevisiae*- α -Faktor ist.
9. Verfahren gemäß Anspruch 2, bei dem das Säugetierprotein ein humaner epidermalen Wachstumsfaktor ist.
- 20
10. Verfahren gemäß Anspruch 4, bei dem Gene* einen humanen epidermalen Wachstumsfaktor kodiert.
11. Verfahren gemäß Anspruch 5, bei dem Gene* einen humanen epidermalen Wachstumsfaktor kodiert.
- 25
12. Verfahren gemäß Anspruch 7, bei dem das heterologe Polypeptid einen humanen epidermalen Wachstumsfaktor einschließt.
13. Verfahren gemäß Anspruch 8, bei dem das heterologe Polypeptid einen humanen epidermalen Wachstumsfaktor einschließt.
- 30
14. Verfahren gemäß einem jeden der Ansprüche 1, 7 oder 8, bei dem das heterologe Polypeptid zum Erhalt eines reifen Polypeptids intrazellulär oder extrazellulär weiterverarbeitet wird.
15. Verfahren gemäß Anspruch 4, bei dem das heterologe Polypeptid zum Erhalt eines reifen Polypeptids intrazellulär oder extrazellulär weiterverarbeitet wird.
- 35
16. Verfahren gemäß Anspruch 15, bei dem das reife Polypeptid aus der von dem Wachstumshormon, Somatomedinen, dem epidermalen Wachstumsfaktor, Insulin, Renin, Calcitonin, Albuminen und Interferonen gebildeten Gruppe ausgewählt ist.
- 40
17. Verfahren gemäß Anspruch 16, bei dem das reife Polypeptid aus von dem Wachstumshormon, Somatomedinen, dem epidermalen Wachstumsfaktor, Insulin, Renin, Calcitonin, Albuminen und Interferonen gebildeten Gruppe ausgewählt ist.
- 45
18. Verfahren gemäß einem jeden der Ansprüche 1, 7, 8 und 15 bis 17, bei dem die Hefe der Stamm AB 103, ATCC-Nr. 20658, ist.

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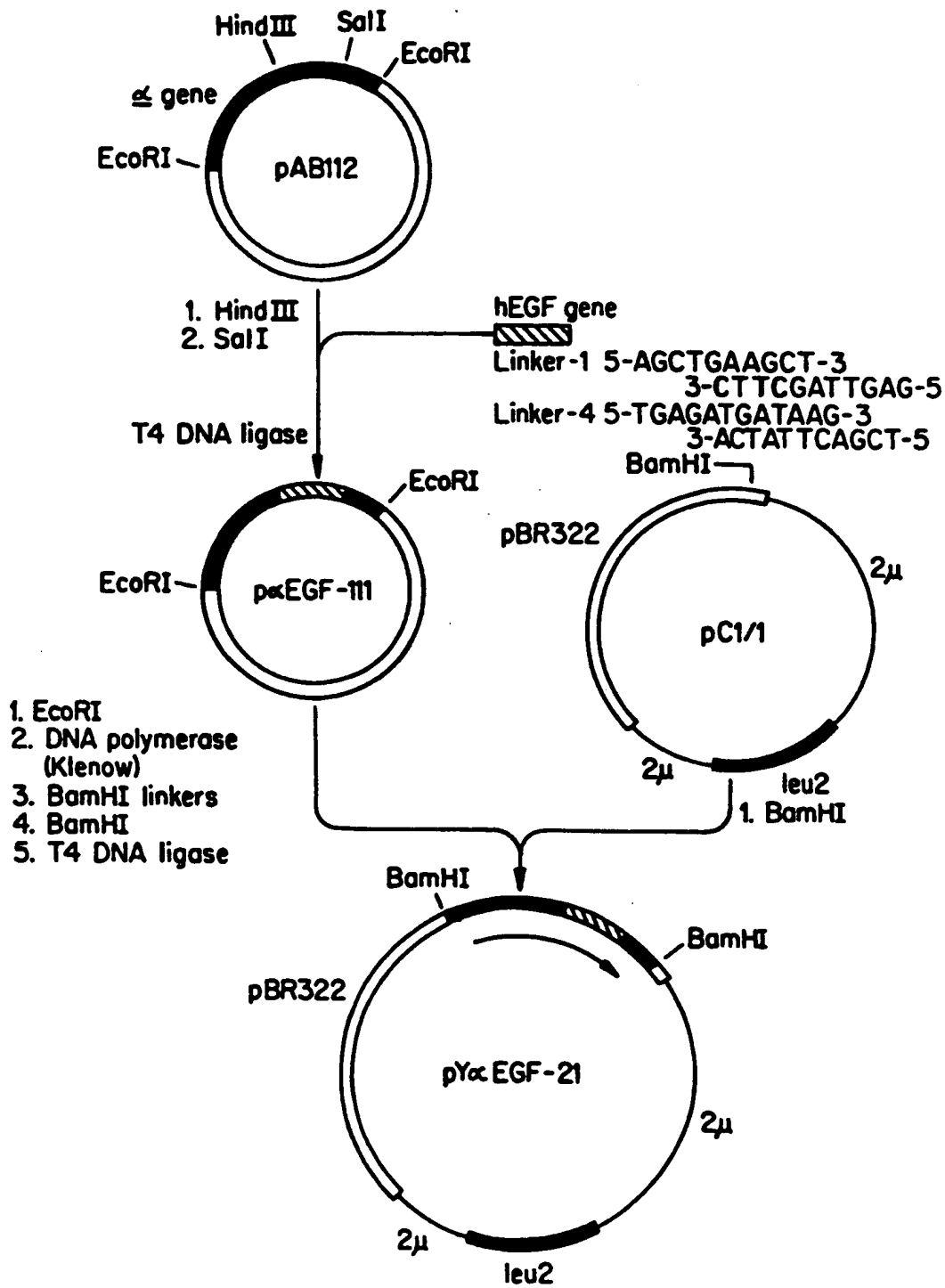


FIG. 1.

80 85 90 5
 Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Glu Ala Asn Ser Asp Ser Glu
 a. GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT AAC GCT AAC TCC GAC TCC GAA
 CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA CTT CGA TTG AGG CTG AGG CTT
 (pY α EGF-21)
 80 85 90 5
 Gly Val Ser Leu Asp Lys Arg Glu Ala Glu Ala Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
 b. GGG GTA TCT TTG GAT AAA AGA GAG GCT GAA GCT TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT AGA AAC CTA TTT TCT CTC CGA CTT CGA AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 (pY α EGF-22)
 80 85 5
 Gly Val Ser Leu Asp Lys Arg Asn Ser Asp Ser Glu
 c. GGG GTA TCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT AGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 (pY α EGF-23)
 80 85 5
 Gly Val Pro Leu Asp Lys Arg Asn Ser Asp Ser Glu
 d. GGG GTA CCT TTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT GGA AAC CTA TTT TCT TTG AGG CTG AGG CTT
 (pY α EGF-24)
 80 85 5
 Gly Val Glu Leu Asp Lys Arg Asn Ser Asp Ser Glu
 e. GGG GTA GAG CTG GAT AAA AGA AAC TCC GAC TCC GAA
 CCC CAT GTC GAC CTA TTT TCT TTG AGG CTG AGG CTT
 (pY α EGF-25)
 50 53
 Trp Trp Glu Leu Arg
 f. TGG TGG GAA TTG AGA TGA TAA GTC GAC CGA TG
 ACC ACC CTT AAC TCT ACT ATT CAG CTG GCT AC

FIG. 2.